Hes·so VALAIS WALLIS
School of Engineering

# Degree Program
# Life Technologies

## Major Biotechnology

# Diploma 2015

*Antsa Rabenifara Estoppey*

*Development of a molecular method for enumerating viable Ascaris eggs from feacal sludge*

*Professor*
Sergio Schmid

*Expert*
Julien Maillard

*Submission date of the report*
14.08.2015

HES-SO Valais-Wallis • rte du Rawyl 47 • C.P. • 1950 Sion 2
+41 27 606 85 23 • hei@hevs.ch • www.hevs.ch

Valais excellence

**HES-SO Valais**

| SI | TV |
|---|---|
| X | X |

# Données du travail de diplôme
## *Daten der Diplomarbeit*

FO 1.2.02.07.AB
pof/31/01/2009

| | | |
|---|---|---|
| ☐ FSI<br>☒ FTV | Année académique / *Studienjahr*<br>**2014/2015** | No TD / *Nr. DA*<br>**bt/2015/37** |
| Mandant / *Auftraggeber*<br>☐ HES—SO Valais<br>☐ Industrie<br>☒ Ecole hôte **EPFL** | Etudiant / *Student*<br>**Antsa Rabenifara** | Lieu d'exécution / *Ausführungsort*<br>☐ HES—SO Valais<br>☐ Industrie<br>☒ Ecole hôte **EPFL** |
| Professeur / *Dozent*<br>**Sergio Schmid** | Expert / *Experte (données complètes)*<br>**Julien Maillard, EPFL ENAC IIE LBE, CH C2 391 (Bâtiment CH), Station 6, CH-1015 Lausanne**<br>**julien.maillard@epfl.ch** | |
| Travail confidentiel / *vertrauliche Arbeit*<br>☐ oui / ja [1] ☒ non / nein | | |

Titre / *Titel*

**Development of a molecular method for enumerating viable Ascaris eggs from feacal sludge**

Description et Objectifs / *Beschreibung und Ziele*

La majorité de la population urbaine des pays en voie de développement dépend de systèmes assainissement autonomes produisant des boues de vidange dont le contenu en pathogènes représente un risque important pour l'environnement et la santé. Le projet du groupe du professeur Holliger de l'EPFL vise à évaluer et optimiser le traitement des boues de vidange par digestion anaérobie. Comme organisme modèle pour cette analyse, les œufs d'helminthe (vers qui infectent les porcs) ont été choisis. Dans le cadre de son travail de diplôme, Mme Antsa Rabenifara aura à établir une méthode moléculaire de quantification des œufs d'helminthe sur la base d'une étude publiée en 2006 par Pecson *et al.* [1] permettant un suivi optimal des pathogènes et de leur viabilité lors des tests d'inactivation.

**Objectifs**

L'objectif général du travail de diplôme est d'établir et d'appliquer une méthode moléculaire (qPCR) pour la quantification des œufs d'helminthe dans des boues de vidange.

Les objectifs spécifiques sont :

1) Elaborer et tester des méthodes d'extraction des acides nucléiques des boues de vidange
2) Mise en place de la méthode de qPCR
3) Développer une procédure d'analyse des échantillons de boues de vidange contenant des quantités connues d'œufs d'helminthe
4) Analyser des échantillons issus de tests d'inactivation par digestion anaérobie
5) Evaluer les résultats de quantification
6) Rédiger un rapport final et présenter de manière orale les résultats

[1] Pecson B.M., Barrios J.A., Johnson D.R. & K.L. Nelson. 2006. A Real-Time PCR Method for Quantifying Viable *Ascaris* Eggs Using the First Internally Transcribed Spacer Region of Ribosomal DNA. Appl. Environ. Microbiol.72, 7864 – 7872

[1] *Par sa signature, l'étudiant-e s'engage à respecter strictement la directive et le caractère confidentiel du travail de diplôme qui lui est confié et des informations mises à sa disposition.*
*Durch seine Unterschrift verpflichtet sich der Student, die Richtlinie einzuhalten sowie die Vertraulichkeit der Diplomarbeit und der dafür zur Verfügung gestellten Informationen zu wahren.*

Rapport reçu le / *Schlussbericht erhalten am* ..... Visa du secrétariat / *Visum des Sekretariats* .....

Délais / *Termine*

Attribution du thème / *Ausgabe des Auftrags:*
27.04.2015

Exposition publique / *Ausstellung Diplomarbeiten:*
28.08.2015

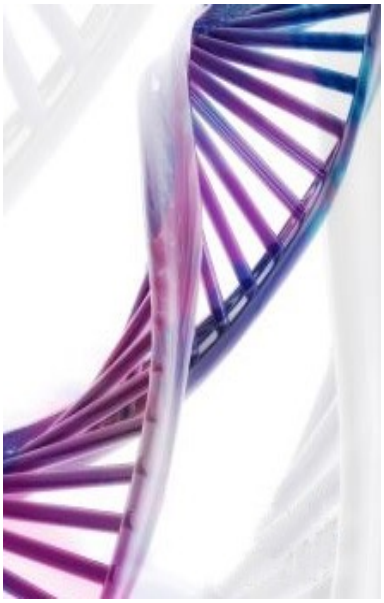
Fin des travaux de diplôme dans les laboratoires de l'école / *Ende der Diplomarbeiten in den Labors der Schule:*
10.07.2015

Défense orale / *Mündliche Verteidigung:*
dès la semaine 35 / *ab Woche 35*

Remise du rapport / *Abgabe des Schlussberichts:*
14.08.2015 | 12h00

Signature ou visa / *Unterschrift oder Visum*

Responsable de la filière
*Leiter des Studiengangs:* .....[1] Etudiant/*Student*: .....



# *Development of a molecular method for enumerating viable Ascaris eggs from sludge*

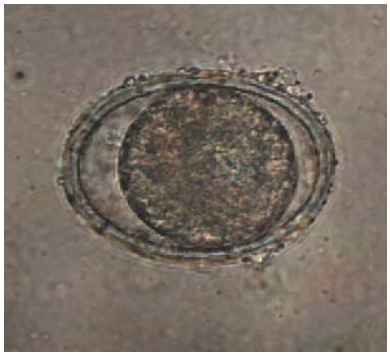
Graduate Rabenifara Estoppey Antsa

## Objectives

This study is a part of a project that aims to set up a molecular method to evaluate the viability of *Ascaris* eggs during sludge treatment. The specific objectives of the thesis is to develop a method for breaking eggshell prior to nucleic acid extraction and to design and optimize a qPCR method to quantify viable *Ascaris* eggs.

## Methods | Experiences | Results

*Ascaris suum* eggs were used as a model for the development of the method. The complexity of *Ascaris* eggshell structure is an obstacle for the nucleic acid extraction. This is why thirteen combinations of thermic, enzymatic, chemical and mechanical treatments prior to nucleic acid extraction were tested. Mechanical treatments were shown to be the most efficient method to break eggshell. Among the four tested RNA extraction methods, the one using PowerLyzer UltraClean Tissue and Cells RNA isolation kit gave the highest extraction yield (16.8 ng/µl) and the purest RNA molecules. The classical reverse tanscription method using anchored oligo-dT primer allowed the best selectivity compared to the two other studied methods.
Three molecular targets were assessed : ITS-1 rDNA as a reference target, As-p18 mRNA encoding for a developmental regulated protein and CDP6 mRNA encoding for a regulator protein involved in cell apoptosis. The primer design was optimized and the linearity ranges of the qPCR methods were evaluated. The LLOQ of the methods quantifying ITS-1 rDNA, As-p18 cDNA and CDP6 cDNA are 33 copies/µl, 17 copies/µl and 3 copies/µl, respectively.



Light microscopy photograph of a single-cell *Ascaris suum* egg.

Light microscopy photograph of a broken *Ascaris suum* egg.

Bachelor's Thesis
| 2015 |

Degree programme
*Life Technologies*

Field of application
*Biotechnology*

Supervising professor
*Dr Schmid Sergio*
*sergio.schmid@hevs.ch*

Partner
*Julien Maillard, LBE-EPFL*
*julien.maillard@epfl.ch*

## Table of contents


**List of abbreviations** ..... **3**

**1. Introduction** ..... **4**
1.1 Objectives ..... 5
1.2 Parasitic pathogens model: *Ascaris* ..... 5
1.3 Enumeration methods ..... 7
1.4 The molecular targets ..... 8
1.4.1 Reference target: ITS-1 rDNA ..... 8
1.4.2 Growth marker: As-p18 protein ..... 9
1.4.3 Apoptosis marker: CDP6 protein ..... 9

**2. Materials** ..... **11**
2.1 Instruments ..... 11
2.2 Softwares ..... 11
2.3 Solutions and powders ..... 11
2.4 Organisms ..... 12
2.5 Oligonucleotides ..... 13
2.6 Tested kits ..... 13

**3. Methods** ..... **14**
3.1 Nucleic acid extraction ..... 14
3.1.1 Eggshell breaking method ..... 15
3.1.2 RNA extraction methods ..... 16
- Egg suspension ..... 16
- Eggs within sludge ..... 17

3.2 Reverse transcription ..... 18
3.3 Conventional PCR ..... 19
3.4 qPCR analysis ..... 21
3.4.1 Plasmids development ..... 21
3.4.2 Standard curves ..... 25
3.4.3 qPCR conditions ..... 26

**4. Results** ..... **27**
4.1 Assessment of the eggshell breaking step ..... 27
4.1.1 DNA quantification by spectrophotometry ..... 27
4.1.2 Molecular analysis ..... 27
4.1.3 Observation of the morphological changes due to mechanical treatments ..... 29
4.2 Evaluation of RNA extraction from *Ascaris* eggs ..... 30
4.2.1 Eggs suspension ..... 30
4.2.2 Eggs within sludge ..... 31
4.3 Optimization of the reverse transcription methods ..... 32
4.3.1 Reverse transcription of As-p18 mRNAs ..... 32
4.3.2 Reverse transcription of CDP6 mRNAs ..... 33
4.4 Primer pair evaluation ..... 34
4.4.1 Primers targeting the development marker: As-p18 ..... 34
4.4.2 Primers targeting the apoptosis marker: CDP6 ..... 35
4.5 Development of the quantification method ..... 35
4.5.1 Standard development for CDP6-x7 ..... 35
4.5.2 Standard curves for the qPCR methods ..... 37
4.5.3 Characterization of the qPCR methods ..... 37

**5. Discussion ..... 38**
5.1 Optimization of the *Ascaris* eggshell breaking method ..... 38
5.2 Optimization of the RNA extraction ..... 39
5.3 Optimization of the RT method ..... 40
5.4 Primer pair selection ..... 40
- Development marker: As-p18 ..... 40
- Apoptosis marker: CDP6 ..... 40

5.5 Evaluation of the qPCR method ..... 40
- Characterization of the methods ..... 40
- Inhibitions evaluation ..... 41

**6. Conclusion and outlook ..... 41**

**Acknowledgements ..... 42**

**Bibliography ..... 43**

**Appendixes ..... 46**

## List of abbreviations

| | |
|---|---|
| bp | Base pair |
| CDP | Cell death protein |
| FABP | Fatty acid binding protein |
| iLBP | Intracellular lipid binding protein |
| ITS-1 | First internally transcribed spacer region |
| n.d. | Non detected |
| NC | Negative Control |
| PC | Positive Control |
| PCR | Polymerase Chain Reaction |
| RT | Reverse Transcription |
| STH | Soil-transmitted helminthiases |
| TAE | Tris-HCl / Acetate / EDTA Buffer |
| | |
| DNA | Deoxyribonucleic acid |
| cDNA | Complementary DNA |
| dsDNA | Double stranded DNA |
| ssDNA | Single stranded DNA |
| mtDNA | Mitochondrial DNA |
| rDNA | Ribosomal DNA |
| | |
| RNA | Ribonucleic acid |
| mRNA | Messenger RNA |
| rRNA | Ribosomal RNA |
| | |
| ULOQ | Upper limit of quantification |
| LLOQ | Lower limit of quantification |
| LOD | Limit of detection |

## 1. Introduction

Poor sanitation is frequently associated with infectious diseases such as hepatitis, polio, typhoid, cholera, and especially soil-transmitted helminthiasis (STH). STHs describe human intestinal infections caused by nematode worms. STHs are mainly dangerous due to their chronic impact on nutritional status, development and quality of life of their host rather than the mortality they cause. It was shown that STHs are responsible of around 6'000 deaths and over 4'000'000 disability-adjusted life years (DALYs). DALYs describe the number of years of potential life lost due to premature mortality and the years of productive life lost due to disability. A billion people living in developing countries are infected with STH. 90% of the cases of infections concern children under 5 years old.

WHO envisions reducing morbidity due to STHs among children (1-14 years old). To achieve this goal, WHO recommends a preventive treatment with anthelminthic drugs. This measure consists on the distribution of these drugs to population at risk and the carrying out of a regular preventive treatment, especially among children. The inconvenient of this approach is that a regular treatment can induce the development of a resistance to anthelminthic in targeted parasites. Thus, improving sanitations could be a solution to prevent the spread of the infections. Indeed, one of the main factors of the spread of infections is the re-use of biosolid waste and wastewater in agriculture without pre-treatment. Appropriate sanitation allows reducing the infiltration of eggs into the environment and then also the contamination of water sources and foods.

A waste treatment is necessary to inactivate pathogens and attempt an acceptable number of active pathogen, according to the WHO guidelines. These guidelines recommend to reach < 1 helminth egg per gram of treated sludge or per liter of treated wastewater [1]. Some studies have suggested that a value < 0.1 helminthes egg per gram of treated sludge is more appropriate when children are directly exposed to the sludge [2].

The Department of Water and Sanitation in Developing Countries (Sandec) of the Swiss Federal Institute of Aquatic Science and Technology (Eawag) has set a project called Partnership for Urban Resource Recovery (PURR) in collaboration with the EPFL. The goal of the project is to evaluate and optimize the treatment of feacal sludge by anaerobic digestion. The PURR project focuses on the situation in Vietnam. In this country, the sludge was thrown outside the town without treatment. In northern Vietnam, sludge are used as fertilizer in agriculture without prior treatment [3]. In order to determine if the treatment is efficient, a microscopic method of enumeration was set up. This method is well established though time-consuming and laborious. Therefore, the aim of this study is to develop a molecular method for the quantification of helminth eggs to enable an optimal follow-up of pathogens during and/or after inactivation. *Ascaris suum* eggs will be used as a model during this study. Indeed, *Ascaris* eggs are very resistant and persistent in environmental conditions and inactivation [4]. Inactivation of *Ascaris* eggs is then the guaranty that all other pathogens are also inactivated.

The strength of the eggshells plays a key role in the resistance of the eggs in the environment and is also one of the main challenges of the molecular method of quantification. The eggshell breaking step prior to nucleic acid extraction has to be optimal in order to enumerate the exact number of eggs. The second challenge is the number of cells per egg. Indeed, in its early stage of development, a fertilized egg is composed by one cell. As its development progresses, the cells divide until larval stage, composed of 600 cells. The exact number of cells in each egg is difficult to evaluate. The third challenge of such a method is the complexity of the matrix. Sludge contains many other organisms and many compounds that can interfere in the molecular analysis (specificity, inhibition…).

## 1.1 Objectives

The objectives set for the Bachelor project are:

1) To optimize the nucleic acid extraction in term of quantity and quality:
In the first part, the eggshells breaking method will be assessed for DNA extraction in a stock solution of eggs. The second part will be focused on RNA extraction from a stock solution of eggs and from eggs within sludge. In the same time, the best conditions for the reverse transcription of RNAs will be evaluated.

2) To design and optimize the molecular analysis of three molecular targets
The first target is the ITS-1 region of the rDNA. This target has already been used for helminth diagnosis. The second target is the *As-p18* mRNA encoding for a protein involved in the development of *Ascaris* eggs. The last target is the *cdp6* mRNA encoding for a protein involved in the regulation of cell apoptosis in *Ascaris* eggs.
The specificity and efficiency of the primers will be assessed. Then, the amplification conditions will be optimized and PCR inhibition will be evaluated.

## 1.2 Parasitic pathogens model: *Ascaris*

Helminthes are a broad kind of intestinal parasitic worms composed of three main families:
- Trematodes, commonly named flatworms because of their flattened form (e.g. *Schistosoma*)
- Nematodes, best known as roundworms (e.g. *Ascaris*, *Trichuris* and the human hookworms)
- Cestodes, also called tapeworms (e.g. *Taenia solium*, the "pork tapeworm").

The most common family present in feaces and the most resistant to environmental conditions is the nematodes. The four major species of nematodes infecting humans are *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus* and *Ancylostoma duodenale*. Ascariasis, infection caused by *Ascaris*, is responsible for half the cases of DALYs, even if the majority of infected individuals do not show any symptom.

Above the 13-16 known species of *Ascaris*, two species are of the highest medical significance: *A. lumbricoides* and *A. suum*, infecting humans and pigs respectively. The two species are morphologically indistinguishable but present some minor differences in their genome [2]. During this project, *A. suum* is used as model due to his specific infectivity to pigs and not to humans.

*Ascaris* eggshells have a complex structure that contributes to the high resistance of *Ascaris* eggs in the environment. Indeed, *Ascaris* eggshells have four layers, from outside to inside [5]:
- an irregular thick layer in the surface constituted by muccopolysaccharides and lipoproteins
- a thin vitelline layer coming from the oocyte that strengthen the first structure
- a strong chitinous layer that contributes to the mechanical and chemical resistance of the eggshell
- a lipoprotein layer that contributes to the restricted permeability of the egg.

The strength of the eggshells makes it difficult to break when extracting nucleic acid for molecular analysis. There is no established method for breaking *Ascaris* eggshell.

Depending on its stages of development, the egg can be composed of 1 to 600 cells. The figure bellow shows the steps of an *Ascaris suum* egg development:

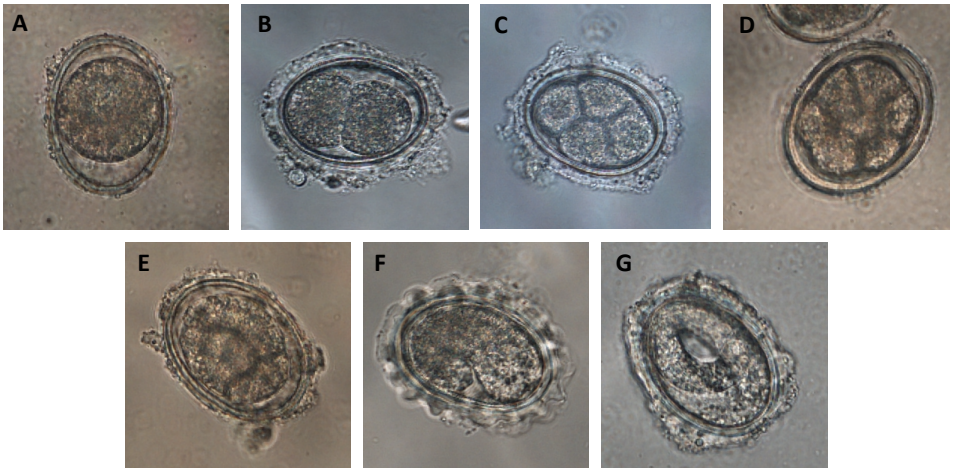



Figure 1: The development steps of Ascaris eggs*: A) single-cell stage – B) two-cell stage – C) four-cell stage – D) eight-cell stage – E) undefined number of cells [8 – 600 cells] – F) prelarval stage – G) larval stage
*The pictures were taken with a 40x objective

The fact is that the eggs shed by one female worm and evacuated in the environment are not in the same stage.

Before attempting the larval stage, *Ascaris* eggs are not infective. It has been assumed that an egg that does not attempt the larval stage within 21 days of incubation in optimal conditions is considered as inactivated.

Ascariasis infection occurs by ingestion of infective eggs via feacal-oral way. Larvae hatch in the small intestine and migrate to the colon. There, they pass through the mucosa and migrate to the liver and the lungs via the portal blood. Once in the lungs, they enter the alveolar space and climb the bronchial tree to attain the throat. Feeling a kind of itching in the throat, the host swallows and the larvae return to the small intestine and reach maturity. Adult worms can live about 6 to 18 months in the intestine. A female can shed about 200’000 eggs per day inside the infected organism [2,6–8]. These eggs are then evacuated outside the host by feaces and develop from single-celled to infective larval eggs in the environment through some stages of cell multiplication and organogenesis. A healthy person could then ingest eggs and the cycle restarts.

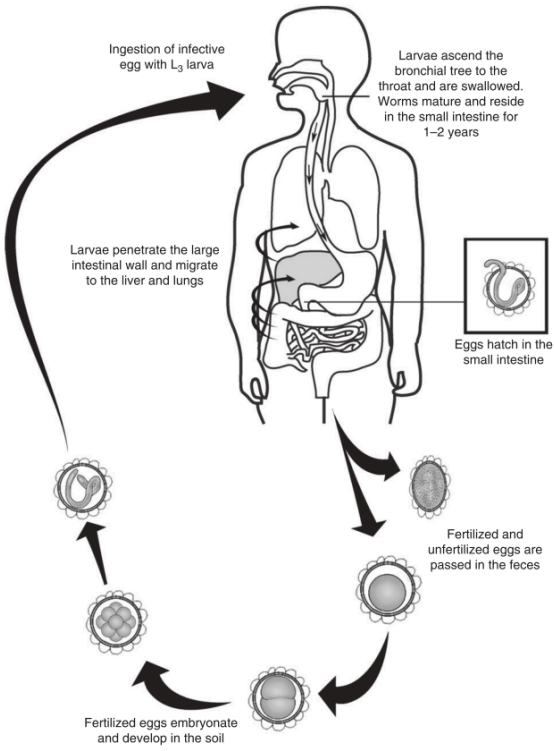



Figure 2: Scheme of the life cycle of *Ascaris lumbircoides* [2]

In a molecular view, *Ascaris suum* has a genome size of 309 kb [9] with 15'399 known coding genes [10]. Wang et al. [10] studied the mRNA dynamics during the development stages of *Ascaris suum* eggs from the first embryonic divisions to the larval stages of the eggs. It has been shown that during the early stages of the egg development, 7626 (49.5 %) genes are significantly expressed in the egg. Thus, in the stage of oocyte, 1177 gene mRNAs are high represented. These mRNAs have been shown to be of maternal contribution and are quickly degraded after fertilization. From one to four-cell stages and from 16 to 256-cell stages, 2962 genes are transcribed. From 4 to 10-cell stage, 1662 genes are specifically exressed. And in the late embryo (larval development), 1826 genes are highly expressed. These datas are of great relevance for the development of a molecular method on *Ascaris* analysis.

## 1.3 Enumeration methods

The most current method for enumerating *Ascaris* eggs is the counting under a light microscopy. The most conventional microscopy method consists of isolating eggs from environmental samples and incubating them at 26°C for 3-4 weeks. Enumeration is then done by microscopy and viable eggs are identified by the presence of motile larva inside the eggs. The advantage of this method is the fact that it is well characterized and easy to implement. The main struggle of this method is the differentiation between viable and inactivated eggs and the differentiation between species. Dead eggs can be identified by the presence of some kinds of bubbles within the egg and by the deformation of cells inside the egg.
Some improvements were proposed in order to moderate this effort. Gaspard et al. [11] proposed to treat the eggs with bleach after the incubation time. This treatment makes the eggshell more transparent so that larvae are easier to distinguish. Combination of microscopy with dying compounds has also been explored [12].

Recently, microscopy method was combined with the viability kit BacLight Live/Dead[13]. This kit was intended to test the viability of bacteria on the basis of the membrane integrity difference between viable and non-viable cells. It has two dye markers: green fluorescent dye (Syto 9) that is taken up by viable cells and a red fluorescence dye (propidium iodide) that enters only non-viable cells and quenches Syto 9. In the case of *Ascaris* eggs, non-viable ones are stained at the inner membrane and the inner contents with both dyes. Viable eggs are stained only at the inner membrane with both dyes. This kind of dying could help to differentiate viable from non-viable eggs and to avoid a long period of incubation prior to enumeration.
One way to improve the specificity and the sensitivity of the method is to adopt a molecular method of enumeration. Moreover, it can decrease the time required to obtain an operable result [2]. Molecular method has already been used for diagnostic purpose, targeting mtDNA or rDNA [14,15]. A qPCR method, targeting the ITS-1 rDNA gene of *Ascaris*, was developed by Pecson et al [7]. This method allows determining the total number of *Ascaris* eggs in one day. For the distinction between viable and non-viable eggs, the method needs 10 days of incubation before DNA extraction. During the incubation time, viable single-cell eggs develop into larval eggs. As a larval egg has about 600 cells, an increase of the qPCR signal by 600 indicates the viability of the eggs. This method is interesting but does not take into account the fact that larvated eggs (partially or fully) can occur in sludge or wastewater.

## 1.4 The molecular targets

To set up a molecular method, it is necessary to determine the fragment of the genome that can provide the appropriate resolution in term of taxonomy and specificity. Indeed, a suitable nucleic acid target should be specific to the target organism and be present in enough quantity to be detected. In this project, three different targets will be assessed:

- ITS-1 rDNA as a reference target
- AS-p18 mRNA as a development marker
- CDP6 mRNA as an apoptosis marker

### *1.4.1 Reference target: ITS-1 rDNA*

The ITS-1 region is a noncoding DNA sequence which is located between the coding sequence for the 18s and 5.8s ribosomal subunit in every eukaryotic genomes. In the case of *Ascaris sp.*, the ITS-1 region has about 450 bp.


Figure 3: Localization of ITS-1 region on rDNA

It was shown that ITS-1 is a region that can be used for taxonomic studies between different groups of parasites including *Ascaris*. The characterization of the rDNA sequences of *Ascaris suum* and *Ascaris lumbricoides* showed that the sequences of 5.8s and the ITS-2 are identical, while the ITS-1 sequence is 1.3% different between both species [13]. The ITS-1 rDNA sequences of *A. lumbricoides* and *A. suum* are available in appendix 1 and appendix 2, respectively.
As mentioned before, the quantification of the ITS-1 rDNA has already been studied in order to enumerate *Ascaris* eggs [7]. To determine the expression level of ITS-1 and thus the bioactivity of eggs, that study worked also on ITS-1 rRNA. But, as ITS-1 is an intergenic region, its RNA has a very short life. The results obtained with this method were therefore not useable. Consequently, other elements were studied to determine the viability of the eggs.

### 1.4.2 Growth marker: As-p18 protein

As-p18 is a protein of the family of fatty acid binding proteins (FABPs). A study was done by Mei et al. [16] to evaluate the occurrence of As-p18 within *Ascaris* eggs and adult worms. The aim of that study was to identify the major proteins in the perivitelline fluid of *Ascaris* eggs and to determine the structure of AS-p18 in order to define its function in the high resistance of the eggs. These proteins could effectively explain the high resistance of the eggshell. The perivitelline fluid was recuperated from infective eggs after mechanical disruption and analyzed by two-dimensional electrophoresis. As-p18 is similar to iLBPs from diverse worms and mammals. The particularity of As-p18 is the presence of a hydrophobic leader sequence and the fact that the proteins are secreted outside the cells. Indeed, iLBPS are intracellular in every organism.

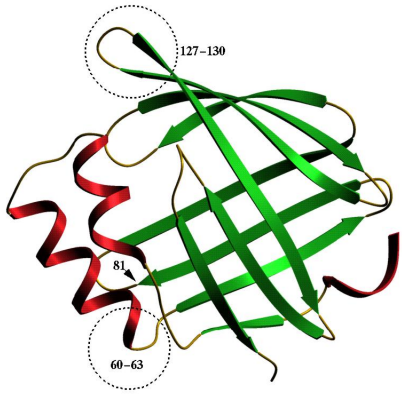


Figure 4: Representation of the protein As-p18 [16]

The function of As-p18 in *Ascaris* eggs is not yet determined precisely, but it was assumed that it contributes to the great resistance of eggs in several hard conditions. They would potentially be responsible of the sequestration of toxic fatty acids and of their oxidation products [15].

Mei et al. [16] identified the protein As-p18 as one of the most abundant in the perivitelline fluid during the early stage of the *Ascaris* eggs devevelopement. It has been shown that the synthesis of As-p18 is under developmental regulation. Thus, the synthesis begins after three days of incubation in favorable conditions. The amount of As-p18 in the perivitelline fluid is at its maximum when the larva reaches the first stage and remains high in its second stage. However, no As-p18 protein was detected in adult organs.

The expression of As-p18 could therefore be used to determine the activity and the viability of *Ascaris* eggs. Indeed, if the eggs were inactivated, no expression would be detected. The way to monitor the expression of As-p18 is the detection and quantification of its mRNA. The As-p18 cDNA and gDNA sequences are available in appendix 3 and appendix 4, respectively.

In this project, two pairs of primers amplifying two different regions on As-p18 mRNA were studied. The first pair amplifies a sequence located between two different exons, while the second one amplifies a sequence located in a unique exon (exon 2). Thus, with the first pair, the sequence obtained from gDNAs is longer than the one obtained from cDNAs (Figure 5).

### 1.4.3 Apoptosis marker: CDP6 protein

The CDP6 (cell death protein) gene encodes for a protein involved in the regulation of cell apoptosis. It is the homolog of CED-6 (Cell Death abnormality 6) gene from *Caenorhabditis elegans* (nematode). The CED-6 gene encodes for an adapter protein involved in the engulfment of apoptotic cells [17]. Indeed, it was shown that, in vivo, cells presenting apoptotic morphology are rapidly engulfed to prevent the releasing of harmful components during apoptosis. There are many proteins involved in the recognition, endocytosis and digestion of corpses and dying cells. The CED-6 protein works downstream CED-1 (a transmembranaire phagocytic receptor) and CED-7 (a transmembranaire ATP-binding cassette protein involved in the recognition of cell-corpse by CED-1) [17–19]. Even if CED-6 has also homologs in mammals and drosophila, it has been shown that all these homologs are similar in function but differ in structure and then also in gene sequence [19]. It is also the case for CDP-6 from *Ascaris.* The CDP6 gDNA sequence is available in appendix 5.

Yu et al. [20] have reported the mRNA expression levels of three genes involved in the development and of two genes involved in apoptosis within *A. suum* eggs under three temperature conditions (20, 50 and 70°C) for 10 days. The studied proteins involved in apoptosis were CDP6 and AIF1. At 20°C, CDP6 expression level had no significant changes over time. At 50°C, the expression level was higher on day 8 while at 70°C, it was higher on day 6. Thus, the higher the temperature is, the earlier are CDP6 and AIF1 expressions within eggs.

In this project, two pairs of primers amplifying two different regions on CDP6 mRNA were tested. One primer pair amplifies a sequence located between the exon 2 and the exon 7. The second primer pair amplifies a sequence located in a single exon (exon 7).

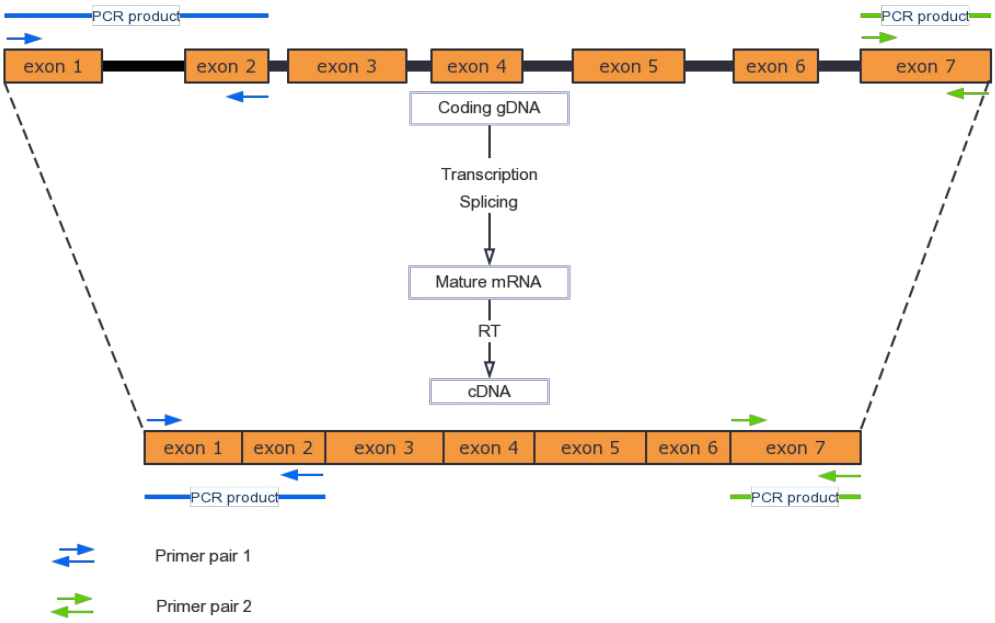



Figure 5: Differences between PCR products from gDNA and cDNA depending on the positions of the primers

# 2. Materials

The instruments (chapter 2.1), the softwares (chapter 2.2), the solutions and powders (chapter 2.3), the organisms (chapter 2.4), the oligonucleotides (chapter 2.5) and the kits (chapter 2.6) used during the project are listed in this chapter.

## 2.1 Instruments

Table 1: List of the instruments used during the project

| Model | Provider | Use |
|---|---|---|
| Professional TRIO Thermocycler | Biometra | PCR and RT |
| RotorGene 3000 | Biometra | qPCR |
| Maxwell 16 | Promega | DNA extraction |
| Ultrasonicator | Branson | Ultrasonication of the eggs before extraction |
| Microscopy Nikon H550L | Nikon | Morphology changes observation |
| Qubit 2.0 Fluorometer | Invitrogen | DNA quantification |
| ND-1000 Spectrophotometer | NanoDrop | DNA or RNA quantification |
| MultiGenius Bio imaging system | Syngene | Gel reading |
| 3130*xl* Genetic Analyzer | AbiPrism | Gene senquecing |

## 2.2 Softwares

Table 2: List of the softwares used during the project

| Software name | Provider | Use |
|---|---|---|
| SeqMan Pro | DNASTAR | Sequencing of the insert of CDP6 in pGEM-T Easy vector |
| Rotor Gene | Biometra | qPCR signals analysis |
| GeneSnap | Syngene | Gel reading |

## 2.3 Solutions and powders

- LB nutrient broth

- Bacto™ Tryptone, BD — Ref. 211705
- Bacto™ Yeast Extract, BD — Ref. 212750
- Bacto™ Agar, BD — Ref. 214010

- LifeGuard™ Soil Preservation Solution, Mobio — Ref. 34861, Cat # 12868-100

- DNase treatment of RNA samples

- RQ1 RNase free Dnase, Promega — Ref. M610A
- RQ1 DNAse 10x Reaction Buffer, Promega — Ref. M198A
- RQ1 DNase Stop solution, Promega — Ref. M199A

- PCR

Taq DNa Polymerase kit, PeqLab Cat # 01-1020

Composition of the kit:
- Reaction buffer 10x, PeqLab
- dNTP-Mix 10 mM each, PeqLab
- $MgCl_2$ , PeqLab
- Taq-DNA Polymerase 5u/µl, PeqLab

- RT

ImProm-II™ Reverse Transcription System, Promega Cat # A3800

Composition of the kit:
- RNasin® Plus RNase Inhibitor, Promega
- ImProm-II™ Reverse Transcriptase, Promega
- ImProm-II™ 5x Reaction Buffer, Promega
- dNTP-Mix 10 mM each
- $MgCl_2$ 25 mM, Promega

- qPCR

- KAPA SYBR® FAST Universal qPCR Kit, KAPA Biosystems KK4602

## 2.4 Organisms

Table 3: List of the organisms used during the project

| Cell line | Provider | Use |
|---|---|---|
| *Escherichia coli XL1-Blue* | LBE | Competent cells (development of plasmids for As-p18) |
| *Escherichia coli DH5α* | LBE | Competent cells (development of plasmids for CDP6) |
| *Ascaris suum* eggs | LBE | Parasitic pathogen model |

The laboratory provided the competent cells. They were stocked at -80°C until use.
The *Ascaris suum* eggs were collected from the uterus of a female worm and stored at 4°C.

## 2.5 Oligonucleotides

Table 4: List of the oligonucleotides used during the project. Microsynth provided all the oligonucleotides.

| Name | Sequence and localization | Length | Target | Product size |
|---|---|---|---|---|
| As-ITS-1-f | 31* 56* 5'-TGCACATAAGTACTATTTGCGCGTAT -3' | 26 | ITS-1 rDNA | 82 bp |
| As-ITS-1-r | 112* 91* 5'-GGCGGCTGACGATAATGTAGT-3' | 21 | | |
| As-p18-f | 252** 271** 5'-GAAAGACACGCATCACAAGG-3' | 20 | As-p18 | 151 bp |
| As-p18-r | 402** 383** 5'-TCTCTACGTCGGTTGGATCG-3' | 20 | | |
| As-p18-x2-f | 147** 166** 5'-GGTGGATCATGAGACAGGTG-3' | 20 | As-p18 exon 2 | 157 bp |
| As-p18-x2r | 317** 298** 5'-TGCTGTCAAGTGCTTCATCC-3' | 20 | | |
| As-CDP6-f | 8409*** 8426*** 5'- ACCTCTGTTACGCCAAGCAT-3' | 20 | CDP6 | 150 bp |
| As-CDP6-r | 9870*** 9852*** 5'- GGAGTGGCAAGTTCTTCGAG-3' | 20 | | |
| As-CDP6-x7-f | 3086*** 3104*** 5'-GGACGAAAGCTCGAGAAC-3' | 19 | CDP6 exon 7 | 162 bp |
| As-CDP6-x7-r | 3240*** 3221*** 5'-TCGTCGTTCATGAAGGTGTC-3' | 20 | | |
| T7 primer | 5'-TAATACGACTCACTATAGGG-3' | 20 | pGEMT vector | |
| SP6 Primer | 5'-ATTTAGGTGACACTATAGAA-3' | 20 | | |
| T25VN | 5'-TTTTTTTTTTTTTTTTTTTTTTTTVN-3' | 27 | mRNAs | - |

(*) *position on the nucleotide sequence in appendix 1*
(**) *position on the nucleotide sequence in appendix 3*
(***) *position on the nucleotide sequence in appendix 5*

## 2.6 Tested kits

Table 5: List of the kits used during the project

| Kit name | Provider | Use |
|---|---|---|
| ReliaPrep™ RNA Tissue Miniprep System | Promega | RNA extraction |
| ReliaPrep™ RNA Cell Miniprep System | Promega | |
| PowerLyzer UltraClean Tissue and Cells RNA isolation kit | MoBio | RNA extraction from complex samples |
| RNA PowerSoil® Total RNA Isolation kit | MoBio | |
| SB Spin PCRapace/Invisorb Fragment CleanUp system | Tratec molecular | Plasmid purification |
| Maxwell 16 Tissue DNA Purification kit | Promega | DNA extraction |
| BioScript All-in-One cDNA Synthesis SuperMix | Biotool | Reverse transcription |

# 3. Methods

In this chapter, the methods required for the nucleic acid extraction (chapter 3.1), the reverse transcription (chapter 3.2), the conventional PCR (chapter 3.3) and the qPCR processes (chapter 3.4) are introduced by a theoretical explanation or/and a literature review (in a blue box).

## 3.1 Nucleic acid extraction

DNA or/and RNA extraction requires a step of cell membrane lysis. The lysis can be done chemically, mechanically, enzymatically or by heating. Once the cell membrane is lysed, the DNA must be purified from all the inner components of the cell (proteins, lipids, etc). In the case of *Ascaris* eggs, it is necessary to break the eggshell prior the cell membrane lysis. Indeed, cells must get out of the shell to be attainable. The step of eggshell breaking is the most challenging part of the nucleic acid extraction.
There is no established method to break *Ascaris* eggshells. The tested methods in this project are taken from diverse researches:

- Leles et al. [14] established a molecular method for the diagnosis of *Ascaris sp*. eggs from feces, targeting the ITS-1 region and *cytb*. In order to break eggshells, eggs samples and feces were exposed to five cycles of heating (100°C) and freezing (liquid nitrogen) before extraction. The method is able to detect as few as 4 eggs per gram of faeces.

- Loreille et al. [21] sequenced three overlapping fragments of the 18S rRNA and one fragment of the *cytb* of ancient *Ascaris* eggs from coprolites at the Middle-Age site of "Places d'Armes" in Namur. Eggshells were broken by ultrasonication for 20 min at 60°C. The samples were then incubated overnight with shaking in the presence 1.5 mg of proteinase K before the DNA extraction.

- The institute of zoology of the University of Zurich set up a protocol for RNA isolation from *Ascaris* eggs and embryos [22]. In this method, the chitinous layer surrounding eggs is removed by washing the sample with bleach.

- Andersen et al. [23] studied the effectiveness of supplementary bead beating for DNA extraction from nematode eggs. They have shown that bead beating with zirconium beads prior to DNA extraction increases the DNA extraction yields from *Ascaris* eggs.

- For most of researches, a kit is used when extracting DNA or RNA without pre-treatment. These kits include most of the time a bead beating step [7,8,16].

The chapter 3.1.1 develops the methods that were tested on the basis of the methods explained above. The chapter 3.1.2 presents the methods that were tested for extracting RNA.

### *3.1.1 Eggshell breaking method*

To insure the shell breaking, some pre-treatments and combination of pre-treatments based on the treatments reported above were tested before the extraction step.

- Heating and freezing cycle

Based on the work of Leles et al. [14], a cycle of heating and freezing was tested during this project. The cycle was set up as follows:

1. Heating in a water bath for 1 minute
2. Freezing in a liquid nitrogen for 30 seconds
3. Heating for 2 minutes
4. Repeat step 2-3 three more times
5. Freezing for 30 seconds

- Proteinase K treatment

A treatment with proteinase K in different conditions was tested. The first condition was set at 60°C for 2h. Two other conditions were set at 37°C for 1h or overnight.

- Bleaching treatment

According to the protocol shared by Spicher A. [22], a wash with 3.8% NaClO was tested. The sample was centrifuged at 2000 x g for 2 minutes and resuspended in 1.5 ml of 3.8% NaClO in a 50 ml falcon tube. It was then incubated for 1 minute at room temperature. After that, the suspension was centrifuged at 700 x g for 2 minutes and resuspended in 1.5ml of 3.8% NaClO. It was necessary to leave the tube in a vertical position for 10 minutes in order to let the bleach acts against the chitinous layer. After 10 minutes, 1.5 ml of water was mixed with the suspension by inverting the tube 3-4 times. The mix was then centrifuged at 700 x g for 2 minutes and the pellet was rinsed 5 times in 3 ml of distilled water.

- Bead beating

The bead beating was done with about 200 mg of 0.1 mm zirconium beads in a 2ml cryo tube. The volume of the sample is completed to 500 µl with deionized water. The sample was then transferred in the cryo tube containing the beads and placed in the Precellys 24 robot. The bead beating was performed at 6500 rpm for two sessions of 45 seconds, separated by 20 seconds of break.

- Ultrasonication

The samples were ultrasonicated at 55 ± 2°C for 20, 40 or 60 minutes.

- Mortar crushing

Crushing was done with a mortar especially designed for eppendorf tubes. The samples were crushed by pushing the mortar inside the eppendorf and by rotating it 5-6 times. The process was repeated 3 times to ensure that all eggs were crushed.

- Suspension in EDTA/N-lauryl sarcosyl

The washed eggs were suspended in 0.5M EDTA with 0.5% N-lauryl sarcosyl in order to dismantle the lipoprotein layer of the *Ascaris* eggshell.

All these treatments were not tested separately. Thirteen combinations of treatment were tested. The Figure 6 shows the combinations tested during this project.

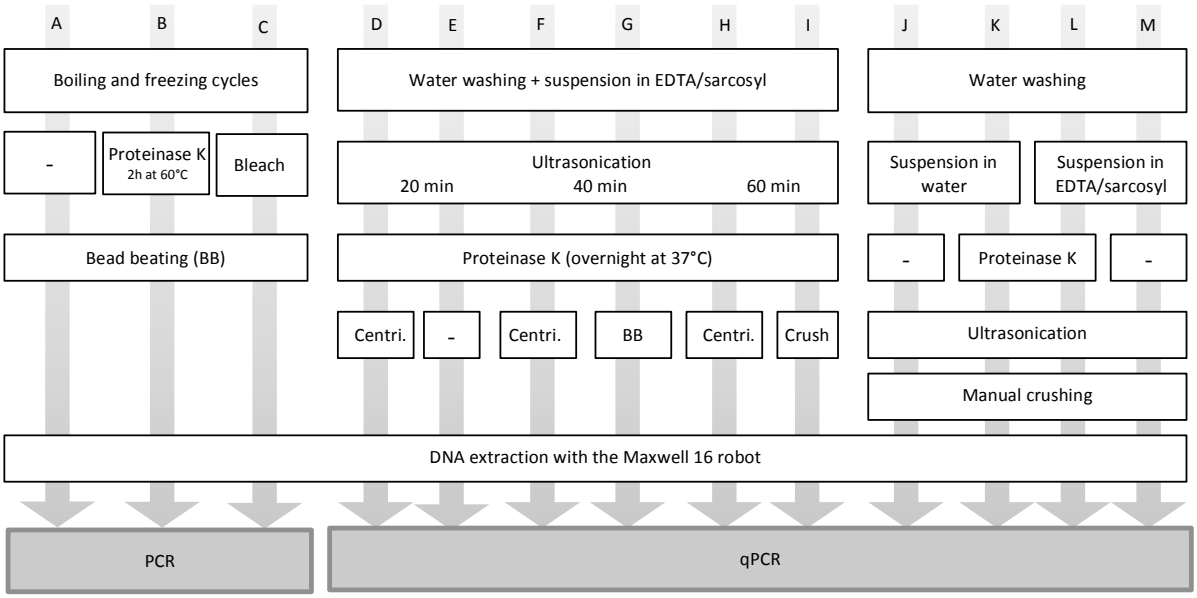



Figure 6: Pre-treatments tested prior to DNA extraction; BB: bead beating, Centri.: centrifugation at 2000xg for 2 min.

After the treatments, the DNAs were extracted using the Maxwell 16 robot with the Maxwell 16 Tissue DNA Purification kit. The DNAs were quantified with the NanoDrop and Qubit HS systems and analyzed by PCR or qPCR targeting ITS-1 region.

The effects of physical treatments (mortar crushing and ultrasonication) were observed by microscopy in order to determine their effect in the morphology of the *Ascaris* eggs.

### *3.1.2 RNA extraction methods*

The viability of a cell can be evaluated by its metabolic activity. The expression level of an RNA transcript is the best molecular way to evaluate the metabolic activity.
RNA extraction was evaluated in purified *Ascaris* eggs and then in *Ascaris* eggs within sludge.

- Egg suspension

Prior testing on sludge, four methods were tested on stock solutions of purified *Ascaris* eggs in order to optimize the extraction in terms of quantity and quality of the extracted RNA.

The first method is the TRIzol-based protocol currently used in the laboratory of environmental biotechnology of EPFL [appendix 6]. Briefly, the protocol is summarized in four big steps:

- Sample lysis and extraction: suspension in TRIzol for the cell lysis and addition of chloroform for the isolation of nucleic acids
- RNA preparation: purification of the RNA (and DNA) with a Zymo column
- DNAse treatment: elimination of the contaminant DNA
- RNA recovery: again a purification of the RNA with a Zymo column

The second method is based on this current protocol, but with a step of mortar crushing before the extraction. The first part of the protocol is as described bellow:

1. Thaw cell suspension (in LifeGuard™) at RT.
2. Centrifuge the sample at 10000 rpm for 5 min at 4°C. Remove the supernatant.
3. Add 0.5 ml of TRIzol.
4. Crush eggshells by rotating a mortar into the tube while pushing down.
5. Incubate sample for 5 min at room temperature for complete lysis.

The rest of the protocol was as described from step 5 (addition of chloroform) in the "sample lysis and extraction" part of the TRIzol-based protocol [appendix 6].

The two last methods are based on two RNA extraction kits: ReliaPrep™ RNA Tissue Miniprep System and ReliaPrep™ RNA Cell Miniprep System. The protocols used during the project are adapted from the protocol provided with these kits [Appendix 7: Reliaprep™ RNA Tissue Miniprep System quick protocol and Appendix 8: Reliaprep™ RNA Cell Miniprep System quick protocol, respectively], except the addition of breaking eggshells step prior to cell lysis. Thus, the first steps of the protocols are the following:

1. Centrifuge the sample at 2'000 x g for 2 minutes
2. Discard the supernatant and resuspend the pellet in 500 µl of LBA + TG Buffer (Tissue) / BL + TG Buffer (Cell).
3. Crush eggs by pushing the mortar into the tube while rotating (8-10x)
4. Clear homogenates by centrifugation for 2 minutes at 2'000 × g, then transfer them to a clean tube.
5. Add 170 µl of Isopropanol. Mix by vortexing 5 seconds.

The next steps were as described in the protocols provided by the kit supplier [appendix 7-8], from step 8 for ReliaPrep™ RNA Cell Miniprep System and from step 6 for ReliaPrep™ RNA Tissue Miniprep System.

- Eggs within sludge

Because of the complexity of the sample matrix, the RNA extraction needs some improvement to reduce inhibitions and RNA degradations. To this end, four extraction methods were tested. The first method is the same TRIzol-based method combined with mortar crushing as the one described above (second method for egg suspension).
The second method is the ReliaPrep™ RNA Cell/Tissue Miniprep System, according to the protocol described in the chapter above (third and fourth method for egg suspension).
The third method is the PowerLyzer® UltraClean® Tissue and Cells RNA isolation kit, especially made for RNA extraction from tissues and complex samples. The extraction was done according to the protocol provided with the kit [appendix 10].
The last method is the RNA PowerSoil total RNA isolation kit, especially made for RNA extraction from cells within complex matrix. The extraction was done according to the protocols provided with the kit [appendix 11].

## 3.2 Reverse transcription

Reverse transcription is a process in which single-stranded RNA is transcribed into complementary DNA (cDNA). This process is done thanks to an enzyme called reverse transcriptase. To make the reaction happens, a primer has to bind to a region of the targeted RNA. The reaction mix has also to contain dNTPs and $Mg^{2+}$ used by the enzyme to synthesize the cDNA. The bound primer is used by the enzyme as a starting point or the reaction.
There are four different types of primer used in RT (cf. figure 7):

- "standard oligo-dT" is a simple sequence made up of nucleotide T
- "anchored oligo-dT" is like a standard oligo-dT excepting the presence of one G, C, or A residue at the 3' end (the anchor)
- "random primer" is six to nine bases long and anneal at different points alongside RNAs
- "gene-specific primer" is designed specifically to target a given RNA sequence

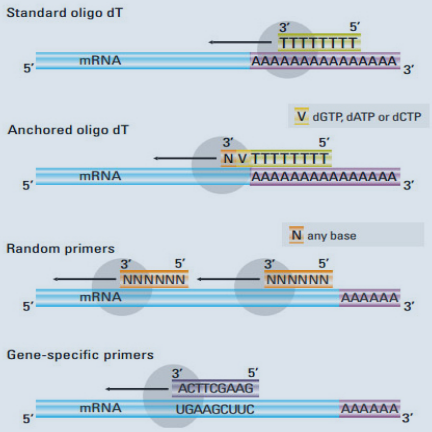



Figure 7: Different types of primer used in RT [33]

The cDNA resulting from the RT reaction is used as a template for the PCR or qPCR reaction.
An RT-PCR (RT followed by a PCR) can be done in two ways:
- In a single step: all reagents necessary for RT and for PCR are added in an unique reaction mixture
- In two steps: the two reactions are performed separately, in two different reaction mixtures

The processing of the results remains the same as for a conventional PCR or a qPCR.

During this project, all RT-PCR or RT-qPCR were done in two-step way.

Three different methods of RT were tested during this project: two classical RT with anchored oligo-dT or gene-specific primer and a kit containing a standard oligo-dT.
For the classical RT, three mixes were prepared separately per sample and per reaction. The mixes are as described below:

Table 6: The composition of the mixes used in classical RT

Mix ARN/primer

| **Reagent** | **Volume [µl]** |
|---|---|
| Primer | 2.5 |
| $H_2O$ | 0.0 |
| RNA sample | 7.5 |
| RNAsin (20 U) | 1.0 |
| **Total*** | **11.0** |

*incubate 5 min at 70°C, then on ice

Mix for RT(+)

| **Reagent** | **Volume [µl]** |
|---|---|
| H2O | 6.1 |
| Buffer 5x | 4.0 |
| dNTPs | 1.0 |
| MgCl2 | 2.4 |
| **ImProm-II RTase** | 1.0 |
| **Total** | **14.5** |

Mix for Rt(-)

| **Reagent** | **Volume [µl]** |
|---|---|
| $H_2O$ | 3.55 |
| Buffer 5x | 2.00 |
| dNTPs | 0.50 |
| $MgCl_2$ | 1.20 |
| **Total** | **7.25** |

Two primer types were tested: the anchored oligo-dT and the gene-specific primer.

A volume of 5 µl of the cold mix RNA/primer was added to the mix for RT(+) while 2.5 µl was added to the mix for RT(-). The samples were then put into a PCR block and the transcription was done with the following conditions:

Table 7: The conditions set up for classical RT

| Reaction | Temperature [°C] | Time [m:s] |
|---|---|---|
| Hybridization | 25 | 05:00 |
| Elongation | 42 | 60:00 |
| Termination | 70 | 15:00 |
| - | 12 | ∞ |

The kit tested in this project was the BioScript All-in-One cDNA Synthesis SuperMix. The supplier of the kit provided the corresponding protocol [appendix 9].

## 3.3 Conventional PCR

A PCR (Polymerase Chain Reaction) is a process that enables the amplification of a DNA molecule. Having multiple copies of a particular sequence of DNA facilitates its analysis. Amplification is done through a thermo stable enzyme named DNA polymerase. This enzyme needs single-stranded DNA as a template to synthesize a new complementary strand. To have the ssDNA, a denaturation of the dsDNA is performed by heating the sample at a temperature close to his boiling point (94-96 ° C). DNA polymerase also needs a short fragment of dsDNA and a free 3'-end as a starting point for the synthesis of the complementary strand. This is why single stranded primers, that are complementary to a known region of the gene of interest, are added to the reaction mixture. The variation of temperature enables the control of the polymerase activity and the binding reaction of the primers. Thus, a PCR cycle is completed in 3 steps:

- Denaturation: heating breaks hydrogen bonds between the two complementary strands of DNA.
- Hybridization: lowering temperature allows the primer to bind to its target sequence on the gene of interest.
- Extension: DNA polymerase uses the hybridized primers as starting point and extends them with the dNTPs, present in huge quantities in the mixture.

This cycle is repeated *n* times, depending on the settings designated by the user. The choice of the number of cycle is based on an equation speculating that the final number of copies of DNA equals $x_0(1 + Ɛ)^n$ - ($x_0$ : initial copy number; Ɛ : efficiency of the reaction).

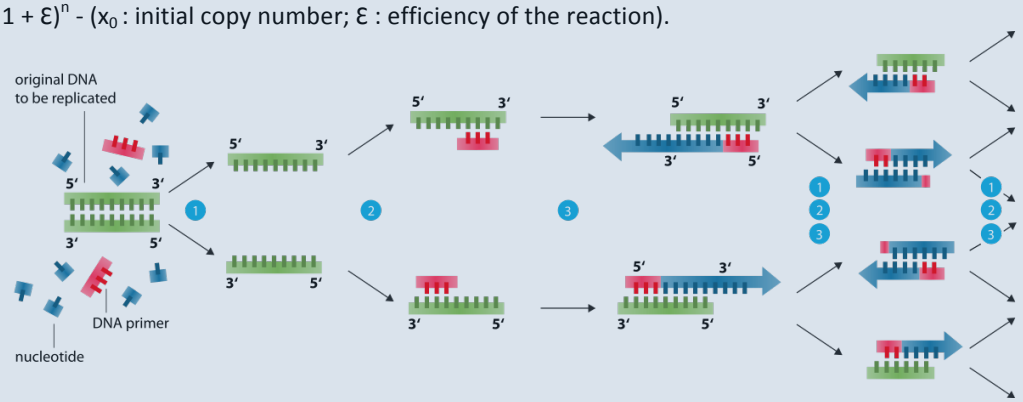



Figure 8: Scheme of a PCR amplification: 1) Denaturation – 2) Primers annealing – 3) Extension [24]

Conventional PCRs were done all along the project. It was executed in order to evaluate the specificity of the primers, the presence or absence of the targets in the sample and the purity of the samples (gDNAs contamination within cDNAs). The mix prepared for each PCR reaction is described in table 8.

Table 8: Composition of the mix used in PCR

| Reagent | Volume [µl] | Final concentration |
|---|---|---|
| Water PCR | 13.5 | - |
| Buffer 10x | 2.0 | 1x |
| dNTPs | 0.6 | |
| MgCl (25 mM) | 0.8 | 1.0 mM |
| Primer 1 (10 µM) | 1.0 | 0.5 µM |
| Primer 2 (10 µM) | 1.0 | 0.5 µM |
| Taq polymerase | 0.1 | |
| **Total** | **19.0** | |

10 µl of the final mix is used for the negative control (without plasmid) and the remaining 9 µl mixed with 1 µl of sample.
The PCR condition used throughout the project is based on the one presented in table 9.

Table 9: The PCR conditions used during the project

| Reaction | Temperature [°C] | Time [m:s] | |
|---|---|---|---|
| Activation | 95 | 05:00 | |
| Denaturation | 95 | 00:45 | 30 cycles |
| Hybridization | 52 | 00:45 | |
| Elongation | 72 | 00:45* | |
| Final elongation | 72 | 05:00 | |

* depending on target size, 01:00 or 02:00

As indicated, the elongation time was set up depending on the target product size.

After a PCR, the samples are analyzed by gel electrophoresis in order to determine if the correct target was amplified. It can also reveal qualitatively the amount of amplified products and the presence of contaminants in the sample. The agarose gel is made with 2% agarose in a 0.5x TAE solution. For 20 ml of solution (agarose in TAE buffer), 2 µl of ethidium bromide is added. The gel migration is performed at 100 V and 500 mA for 40 minutes.
The result is compared to the ladder of DNA molecular weight presented in figure 9.

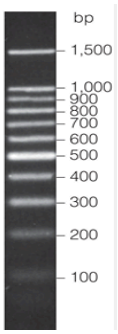



Figure 9: The ladder of DNA in 2% agarose gel in 5x TAE

## 3.4 qPCR analysis

A conventional PCR is a qualitative or semi-quantitative method. To quantify a given DNA target, the quantitative real time PCR or qPCR is more appropriate. This method has the same principles as the conventional PCR presented above excepted that, on qPCR, a fluorescent compound is added to the reaction mix. This compound produces a signal that is proportional to the amount of amplicon after each cycle.
Two types of fluorescent compound are used in qPCR: intercalating agents (ex: SYBR Green I) and fluorescent probes (ex: probe TaqMan...). Intercalating agent binds to dsDNA, between the strands and thus emits fluorescent signal. The fluorescent probes involve a specific complementary DNA sequence that binds to a specific region in the target sequence.
The more commonly used fluorophore is the SYBR Green I. This is due to the fact it is not expensive compared to the specific probes. Indeed, there is no need to design and synthetize an extra specific probe. However, it also means that as it is not specific, the fluorescence can be distorted by the formation of non-specific products during the reaction.
The correlation between the amplification product and the intensity of the fluorescence is then used to calculate the amount of DNA of interest. For this, an important parameter is the cycle threshold or Ct (Cycle threshold): point from which fluorescence is considered to be statistically significant (above the background noise and baseline) . The threshold signal is dependent on the initial amount of DNAs.

As the aim of the project is to detect and quantify the eggs, it is necessary to set up a quantitative method (qPCR) and a calibration curve. The calibration curve is done with linearized plasmids containing a part of the target. Pecson et al. provided the plasmid standard (pASI) used for quantifying the ITS-1 region. Mei et al. provided a small amount of plasmid standard (pASP) containing a fragment of the gene encoding for As-p18. The plasmid standard (pASC) for quantifying CDP6 cDNA was set up from an *Ascaris suum* gDNA extract.

The chapter 3.4.1 develops the methods used to increase the copy number of pASP and to set up the plasmid pASC. The chapter 3.4.2 describes the methods for preparing the plasmids for the standard curves. The chapter 3.4.3 presents the conditions of temperature and duration of each step of the qPCR method.

### *3.4.1 Plasmids development*

- As-p18 plasmid:

The plasmid containing As-p18 DNA fragment (pASP) was provided in small amount. Therefore, it was necessary to increase the copy number of plasmid by cloning it in a competent cell.

The transformation of the competent cells (*E. coli XL1-Blue*) was performed as described in the protocol below:

- Take the tubes containing the competent cells from the freezer and leave them on ice for 5 min
- Prepare two plates NA agar with 50 µg/ml ampicillin
- Add 50 µl of competent cells in two different tubes
- Add pASP to one of the tubes containing the competent cells – mix by inverting the tubes three times and incubate on ice for 15 min
- Put the tubes into a heating block set to 42°C for 1 min and replace them immediately on ice for 3 min
- Add 950 µl of LB broth to the cell samples and incubate for 1h at 37°C and 160 rpm
- Centrifuge at 10000G for 1 min and discard the supernatant (leave about 100 µl in the tube)
- Plate the cells on NA agar – incubate at 37°C overnight

The colonies on the agar plate are the colonies of transformed cells. One colony from the plate is then picked up and cultivated in 10 ml of LB nutrient broth at 37°C overnight.

The plasmids were extracted with the QIAprep Spin Miniprep Kit, according to the protocol provided with the kit [appendix 11].

- CDP6 plasmid

The As-CDP6-x7 primer pair was used to obtain 100 µl (5.8 ng/µl) of the fragment of the CDP6 coding DNA by PCR. The PCR was done with a DNA extract obtained by the extraction method "L" (described in chapter 3.1.1). A control was done by agarose gel to ensure that the amplified product had the right size and that there was no contamination in the sample that could induce a false positive result.
The amplified product was then purified with the SB Spin PCRapace/Invisorb Fragment CleanUp system. According to the protocol provided with the kit, 500 µl of Binding Buffer was added to the PCR sample and mixed by vortexing. The mix was then completely transferred onto a Spin Filter and centrifuged for 2 min at 11.000 x g. Three minutes of centrifugation were added after removing the filtrate. To elute the PCR fragment, the spin filter was placed into a new receiver tube and 20µl of dd$H_2O$ were added directly onto its center. The tube was then incubated 1 min at room temperature and centrifuged at 11'000 x g (11'000 rpm) for 1 min.

*Ligation of the CDP6 DNA fragment in pGEM-T vector:*
The pGEM-T Easy system was chosen for the cloning because of its ease of use. The vectors (Figure 10: pGEM-T Easy vector map) were provided opened (by the restriction enzyme EcoR V) and with terminal thymidines added to both 3'end. This allows to prevent recirculation of the vector and to make a compatible overhang for the product that has to be ligated. The vector contains T7 and SP6 RNA polymerase promoters on both sides of a multiple cloning site within the lacZ region. The presence of $Amp^r$, a coding gene for the resistance to ampicillin, is used as a selection tool after the ligation. The figure 10 represents the map of the vector pGEM-T Easy provided by Promega.

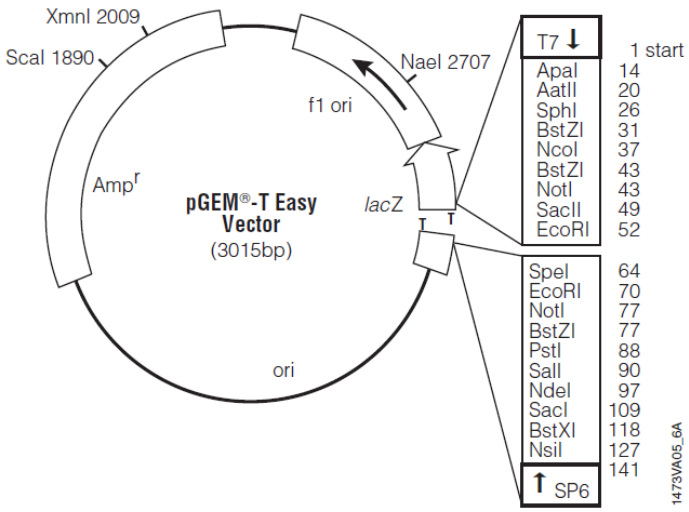



Figure 10: pGEM-T Easy vector map [25]

Thus, before the ligation step, an A-tailing on the 3' extremity of the PCR products has to be done. The protocol is as follows:

1. Start with 7µl (~40 ng) of purified PCR fragment
2. Add 1 µl Taq DNA Polymerase 10X Reaction Buffer
3. Add dATP to a final concentration of 0.2mM → 1 µl dATP à 2 mM
4. Add 5 units of Taq DNA Polymerase (1 µl)
5. Incubate at 70°C for 30 minutes.

The protocol for the ligation is provided with the system [appendix 12].

*Transformation of competent E.coli DH5α:*

The different steps of the protocol of transformation of the competent cells were the following:

- Take the tubes containing the competent cells (*E. coli DH5α*) from the freezer and put them immediately on ice for 5 min
- Prepare three plates NA agar with 100 µg/ml ampicillin
- Prepare three Eppendorf tubes with 50 µl of competent *E. coli DH5α*
- Transfer the plasmid containing CDP6 DNA fragment to one of the tubes and the plasmid without the CDP6 DNA fragment in a second tube – mix by inverting the tubes three times and incubate on ice for 20 min
- Put the tubes into a heating block set to 42°C for 1 min and then immediately replace them on ice for 3 min
- Add 950 µl of LB broth and incubate for 1h at 37°C and 180 rpm
- Centrifuge at 10'000 x g for 1 min and discard the supernatant (leave about 100 µl in the tube)
- Plate the cells on NA agar and incubate at 37°C overnight

*Colony PCR and selection:*

It is necessary to verify if the negative control is negative and if there are less colonies on background control than on transformation plate. When all these controls are done and give the right result, a colony PCR is performed and the right colony is used for the rest of the experience.

The colony PCR is performed according to the protocol described below:

- Prepare a plate LB agar with 100 µg/ml ampicillin
- Prepare 15 PCR tubes numbered 1 to 15 and add 10 µl of sterile water in each tube
- Draw 16 squares in the back of the plate (4x4) and number each square (1-16)
- Choose an isolated colony, pick it up with a sterile toothpick and set the picked colony out in one square
- Rinse the toothpick in the corresponding number of PCR tube
- Repeat the operation with 14 isolated colonies
- Incubate the plate at 37°C overnight
- Heat the PCR tubes at 95°C for 5 minutes in order to break cells
- Prepare a PCR mix as described in the table:

Table 10: The mix prepared for the colony PCR

| Reagent | Volume [µl] |
|---|---|
| Water PCR | 121.5 |
| Buffer 10x | 18.0 |
| dNTPs | 5.4 |
| MgCl (25 mM) | 7.2 |
| Primer T7 (10 µM) | 9.0 |
| Primer SP6 (10 µM) | 9.0 |
| Taq polymerase | 0.9 |
| **Total** | **171.0** |

- For each picked colony, prepare a PCR tube with 9 µl of mix and 1 µl of the heated sample. A negative control is performed with the mix and no sample
- Perform a conventional PCR with an elongation of 1 minute

The result was analyzed in a 2% agarose gel in TAE 0.5x in order to see which colony has the right plasmid. Colonies containing the right colony are then picked up and cultivated overnight in 10 ml of LB nutrient at 37°C and 180 rpm.
The plasmid extraction was done with the QIAprep Spin Miniprep kit according to the protocol provided with the kit [appendix 11]

*Sequencing:*
The extracted plasmids have to be sequenced in order to verify if the plasmid has the sequence corresponding to the target one.

Several steps are necessary before the sequencing step.
The first step is the normalization of the plasmid concentration to 200 ng/µl. The second step allows preparing the sequencing step by amplifying the region of interest. This region is localized between the SP6 and the T7 regions of the plasmid. The amplification is done with the primers SP6 and T7 with the reaction mix shown in the table 11.

Table 11: The mixes prepared for each primer in order to sequence the insert

| Reagent | Volume |
|---|---|
| H2O | 1.4 |
| Mix V3.1 | 2.0 |
| Primer | 1.6 |
| Plasmid | 5.0 |
| **Total** | **10.0** |

One mix per primer and per reaction is prepared. Then, 5 µl of plasmide [200 ng/µl] is added to 5 µl of mix and the PCR reaction can be performed. The PCR conditions are as follows:

Table 12: The PCR conditions for the preparation of the sample for sequencing

| Step | Temp. [°C] | Time [m : s] | |
|---|---|---|---|
| Preheating | 94 | - | |
| Activation | 94 | 01 : 40 | |
| Denaturation | 94 | 00 : 30 | 24 cycles |
| Hybridization | 60 | 00 : 30 | |
| Elongation | 60 | 04 : 00 | |
| --- | 72 | 10 : 00 | |

- Prepare 4 Eppendorf tubes with 64 µl of EtOH 100% each
- Add 26 µl of milliQ water to the PCR products and transfer the mix to the tubes containing the EtOH
- Vortex the tubes
- Incubate for 20 minutes at room temperature in the dark
- Centrifuge for 20 minutes at 13200 rpm and remove the supernatant
- Resuspend in 250 µl of EtOH 70%
- Centrifuge for 10 minutes at 13200 rpm and remove the supernatant

- Let the pellets dry for 20 minutes by inverting the tubes under the hood
- Add 10 µl of formamide in each tube
- Incubate for 2 minutes at 95°C then on ice for 3 minutes
- Vortex and centrifuge for 5 seconds

Once these steps done, the samples are ready for sequencing. The sequencing itself is made by the apparel 3130*xl* Genetic Analyzer, from AbiPrism. The result was treated with the software SeqMan.

### *3.4.2 Standard curves*

- Plasmids digestion:

The restriction enzyme chosen for the digestion of pASI and pASP is the NcoI, whereas pASC was digested with ScaI. The NanoDrop quantification of the plasmid samples gave a concentration of 255 ng/µl for pASI, 70 ng/µl for pASP, and 200 ng/µl for pASC. The mixes prepared for the digestions of the plasmids are presented in table 13.

Table 13: The mixes prepared for digesting plasmids

| | **pASI** | **pASP** | **pASC** |
|---|---|---|---|
| Water PCR [µl] | 4.5 | 3.0 | 3.5 |
| RE Buffer 10x [µl] | 1.0 | 2.0 | 1.0 |
| Plasmid (1000 ng)[µl] | 4.0 | 14.0 | 5.0 |
| NcoI enzyme 10 U/µl [µl] | 0.5 | 1.0 | - |
| ScaI enzyme 10 U/µl [µl] | - | - | 0.5 |
| **Total [µl]** | **10.0** | **20.0** | **10.0** |

These preparations were incubated for 2h at 37°C.
After the digestion, it is necessary to dephosphorylate the extremities of the linearized DNA. Therefore, 1 µl of SAP (shrimp alkaline phosphatase, Takara Lot #N701D8) is added to the digested sample and the mix is incubated at 37°C for 30 min. The dephosphorylating step is done twice for each plasmid sample.

- Purification and quantification of the linearized plasmid:

The purification of the linearized plasmid is done with the QIAquick PCR Purification kit according to the provided protocol [Appendix 14: .

The quantification of the purified plasmids was made with the fluorescent system Qubit BR. The robot gives a concentration of DNA in ng/µl and it has to be converted in number of copies/µl. The equation used for this conversion is as follows:

$$\#\text{copies}/\mu\text{l} = \frac{\text{C} \times 10^{-9}}{MW_{plasmid}} \times N_A$$

where:

| | | |
|---|---|---|
| C | average of the concentration values of DNA given by Qubit | [ng/µl] |
| $MW_{plasmid}$ | mass weight of the plasmid | [g/mol] |
| $N_A$ | Avogadro number | = $6.022 \times 10^{23}$ $mol^{-1}$ |

The molecular weight of a base pair is set as equal to 650 g/mol.

### *3.4.3 qPCR conditions*

For the qPCR reactions, the reaction mix is as described in table 14 for each reaction tube.

Table 14: The mix prepared for each tube reaction when performing a qPCR

| Reagent | Volume [µl] |
|---|---|
| $ddH_2O$ | 2.1 |
| Forward primer | 0.2 |
| Reverse primer | 0.2 |
| KAPA mix | 5.0 |
| **Total** | **7.5** |

2.5 µl of the sample to be analyzed is then added to the mix directly inside the qPCR tube. The conditions of the qPCR are as described in the table below.

Table 15: The qPCR conditions used during the project

| Step | Temp. [°C] | Time [m : s] | |
|---|---|---|---|
| Preheating | 95 | - | |
| Activation | 95 | 10 : 00 | |
| Denaturation | 95 | 00 : 10 | 40 cycles |
| Hybridization | 60 | 00 : 15 | |
| Elongation | 72 | 00 : 20* | |
| --- | 72 | 00 : 45 | |
| | 72 - 95 | 1°C each 5 sec | |

* for ITS-1 analysis: 00:08

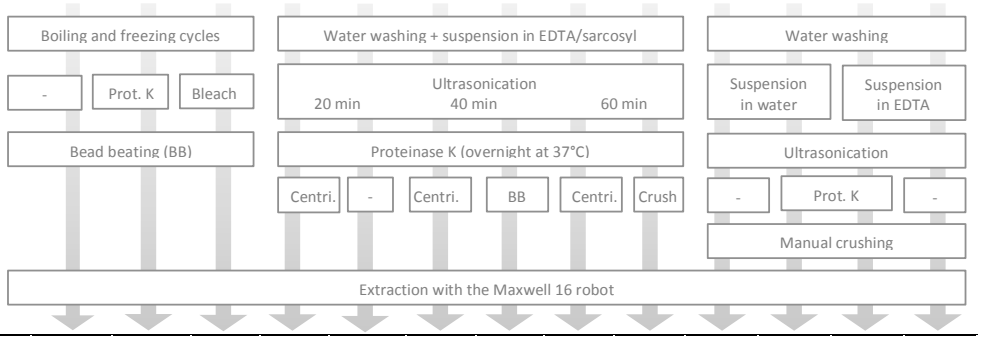
# 4. Results

## 4.1 Assessment of the eggshell breaking step and analysis of the resulting extracted DNA

Thirteen treatments were tested prior to DNA extraction in order to break eggshells and release the inner cells of the eggs. The extracted DNAs were quantified by absorbance measurement with NanoDrop and Qubit systems and analyzed by PCR or qPCR, targeting ITS-1.

### *4.1.1 DNA quantification by spectrophotometry*

The extracted DNAs were quantified by NanoDrop and Qubit HS systems and the results are given in the figure 11.




| Treatment | A | B | C | D | E | F | G | H | I | J | K | L | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial volume [µl] | 300 | 300 | 300 | 150 | 150 | 150 | 150 | 150 | 150 | 250 | 250 | 250 | 250 |
| NanoDrop [ng/µl] | 4.2 | 5.5 | 1.4 | 0.5 | 34.1 | 3.9 | 1.1 | 2.3 | 2.8 | 1.1 | 0.9 | 1.5 | 1.9 |
| Qubit HS [ng/ml] | 29.6 | 52.0 | 1.7 | 1.6 | 3.4 | 2.0 | 1.8 | 2.4 | 3.4 | 63.7 | 22.0 | 83.1 | 23.6 |

Figure 11: The results of the quantification of the extracted DNA by absorbance measurement

There are huge differences between the results obtained with NanoDrop and Qubit for the same sample. The highest difference is for the treatment J, where the concentration obtained with Qubit is 58x the concentration obtained with NanoDrop.
The treatment L gives the higher amount of extracted DNA, followed by the treatment J and B.

### *4.1.2 Molecular analysis*

The samples extracted with the treatment A, B and C were analyzed by PCR, targeting the ITS-1 region. Diluting the samples before the PCR allowed assessing the presence of inhibitors. The figure 11 shows the result of the PCR.

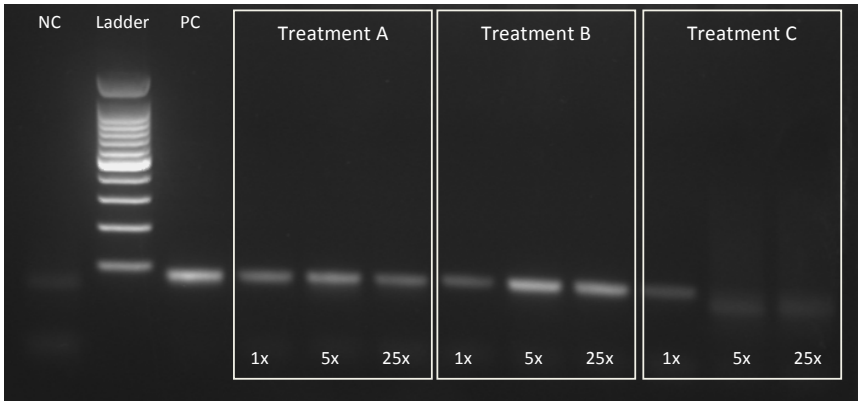



Figure 12: The agarose gel of the PCR products targeting ITS-1 in samples treated with treatment A, B and C prior to DNA extraction - the samples were diluted 5x and 25x

The agarose gel shows that the NC is contaminated, but the difference between the signals observed in the NC and the samples is sufficient to consider the result as useable.
For the treatment A, there is no significant change between the signals obtained with the non-diluted and diluted samples. The treatment B shows a stronger signal with the samples diluted 5x and 25x compared to the signal of the non-diluted sample. The treatment C does not show a more significant signal compared to the NC. The result of the quantification shows that the poor signal is due to the fact that the concentration of DNA was too low.

The rest of the samples were analyzed by qPCR, targeting the ITS-1 region. Some dilutions were done before the qPCR analysis in order to assess the presence of inhibitors.

Table 16: The results of the qPCR targeting ITS-1 on samples treated with treatment D to M. The values obtained with diluted samples have already been corrected by the dilution factor

| **Dilution** | **Number of copies obtained with treatments D to M [$10^5$ copies/µl]** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** |
| 1x | n.d. | n.d. | n.d. | n.d. | n.d. | 3.93 | - | - | - | - |
| 2x | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - | - | - | - |
| 5x | 0.59 | n.d. | 0.32 | 0.12 | 0.98 | 2.46 | 0.32 | 0.67 | 1.17 | 0.04 |
| 10x | 0.83 | 0.87 | 0.76 | 0.17 | 1.32 | 5.31 | 0.14 | 1.04 | 0.83 | 0.23 |
| 20x | 0.84 | 0.82 | 1.07 | 0.61 | 1.48 | n.d. | 0.13 | 0.67 | 0.74 | 0.16 |
| 40x | 0.85 | 1.24 | 1.49 | 0.32 | 1.27 | 5.38 | 0.26 | 0.77 | 0.95 | 0.26 |
| 80x | 0.80 | 1.34 | 1.34 | n.d. | 1.65 | 5.82 | 0.11 | 0.42 | 0.68 | 0.29 |
| **Average** | **0.83** | **1.29** | **1.30** | **0.61** | **1.43** | **5.50** | **0.19** | **0.78** | **0.80** | **0.26** |

According to these results, a dilution of 10x or 20x is sufficient to eliminate the inhibition effect of the matrix.

### *4.1.3 Observation of the morphological changes due to mechanical treatments*

After the ultrasonication, 50 µl of sample were collected and spread in the microscopic slide. The slide is then closed and sealed with nail polish. The effects of the ultra-sonication in eggshell were observed by light microscope. The images below show the changes observed after ultrasonication.

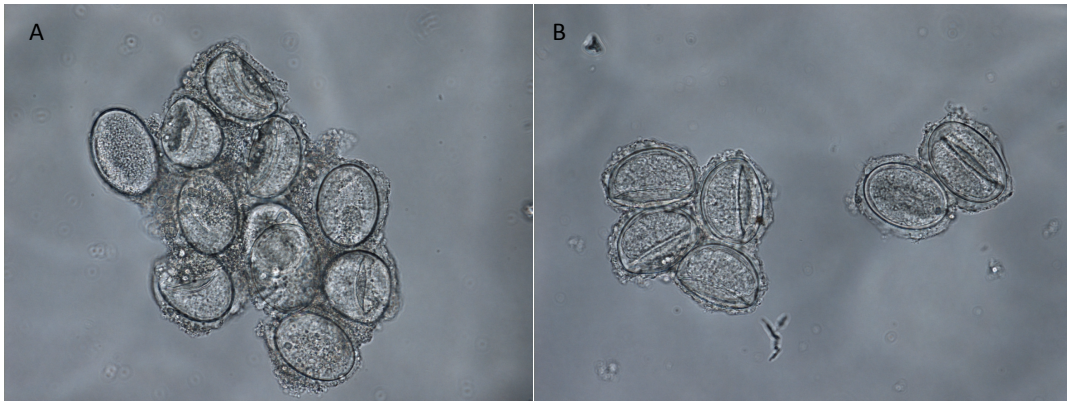



Figure 13: Microscopy observation of the effect of the ultrasonication on the *Ascaris* eggshells

After ultrasonication, the majority of the eggs presented a line in their middle. There were some eggs that remained unbroken, mostly within the sample sonicated for 20 minutes. In the figure 13A, it can be noticed that some cells surround the eggs.

After the mortar crushing, 50 µl of sample were collected and spread in the microscopic slide. The slide is then closed and sealed with nail polish. The effects of the crushing in eggshell were observed by light microscope. The images below show the changes observed after the mortar-crushing step.

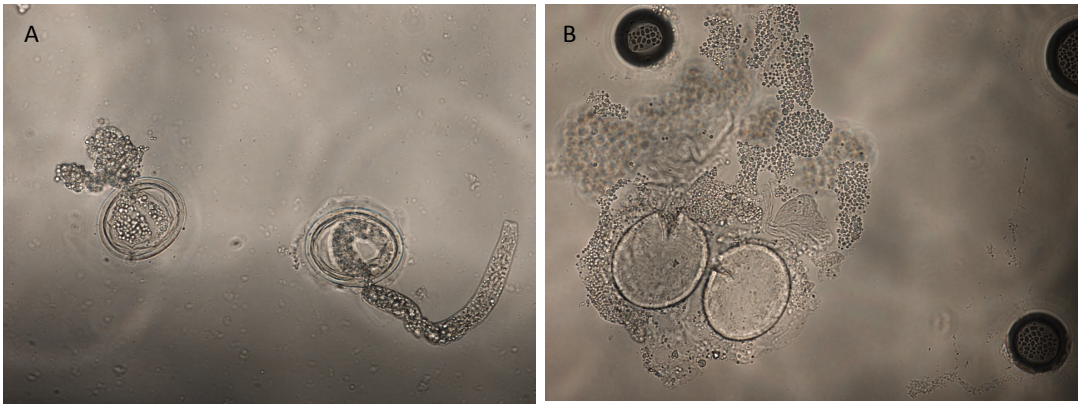



Figure 14: Microscopy observation of the effect of the mortar crushing on the *Ascaris* eggshells

The figure 14A shows what happens to the majority of the eggs. The eggshell is not totally broken but enough for freeing a part of the inner cells or a part of the larva. The case in the figure 14B is less abundant although it is what is expected with this kind of treatment.

## 4.2 Evaluation of RNA extraction from *Ascaris* eggs

### *4.2.1 Eggs suspension*

Three different extraction protocols were tested. The first protocol is based on the Reliaprep™ RNA Cell Miniprep System. The sample extracted with this kit was named "kit C". The second protocol is based on the Reliaprep™ RNA Tissue Miniprep System. The sample extracted with this kit was named "kit T". The last protocol is based on the TRIzol method of extraction. The sample extracted with this method was named "TRIzol".

The extracted RNA were quantified with NanoDrop. Each extraction was done in duplicate. The results are presented in the table below.

Table 17: The concentration of RNAs extracted with the Reliaprep™ RNA Cells/Tissue Miniprep System and the TRIzol based method

| | **Kit C** | **Kit T** | **TRIzol** |
|---|---|---|---|
| Extraction A [ng/µl] | 3.3 | 2.4 | 2.9 |
| Extraction B [ng/µl] | 2.6 | 2.5 | 2.3 |
| **Average [ng/µl]** | **3.0** | **2.5** | **2.6** |

There are no significant differences between the results obtained with the three methods.
A PCR targeting As-p18 is a good way to know if the samples are contaminated with DNA. With the couple of primers As-p18-f and As-p18-r, it is possible to differentiate the gDNA fragment from the cDNA fragment. Indeed, these primers amplify 151 bp when amplifying cDNA whereas they amplify about 1000 bp when amplifying gDNA.

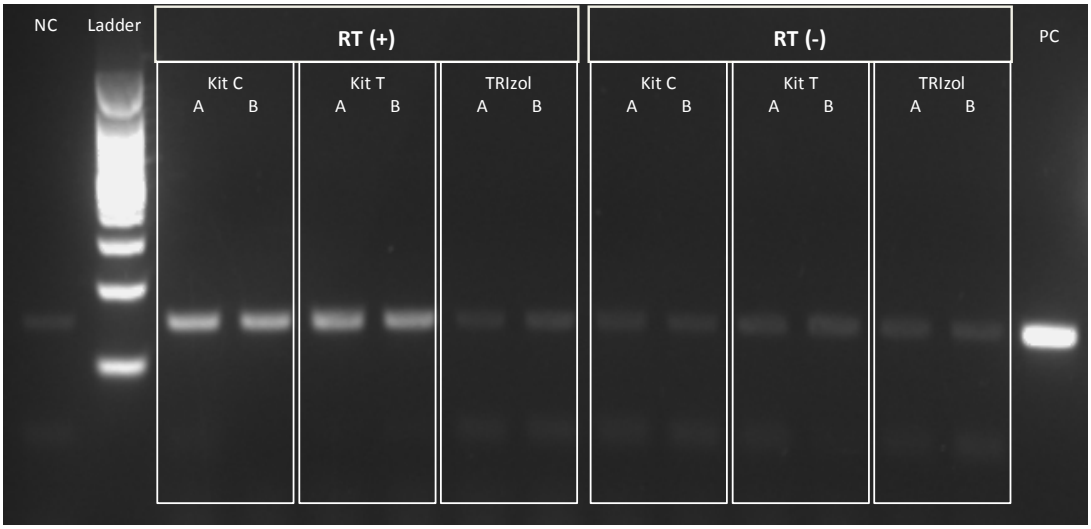



Figure 15: PCR products of the cDNAs obtained from *Ascaris* egg suspension with three different extraction methods

The RT(-) samples show no contamination with gDNA and there is no signal around 750 bp. Both RT methods show fluorescent signals at the expected size.

### *4.2.2 Eggs within sludge*

Four extraction methods were tested to extract RNA from complex samples. Three methods are made with kits from different providers and the last one is the chemical TRIzol-based method. The table below shows the results of the quantification of the RNE extracted with the four methods.

Table 18: Quantification of the extracted RNA from the *Ascaris* eggs within sludge

| Sample | Kit/method | Concentration [ng/µl] | A260/A280 | A260/A230 |
|---|---|---|---|---|
| **PowerLyzer** | PowerLyzer UltraClean Tissue and Cells RNA isolation kit + DNAse treatment | 16.8 | 1.97 | 1.12 |
| **ReliaPrep** | ReliaPrep™ RNA Tissue/Cell Miniprep System | 6.7 | 1.61 | 1.09 |
| **PowerSoil** | RNA PowerSoil total RNA isolation kit + DNAse treatment | 6.3 | 1.52 | 0.53 |
| **TRIzol** | TRIzol based RNA extraction protocol | 27.2 | 1.45 | 0.54 |

For pure samples, the A260/A280 and A260/A230 ratios values are expected to be around 2.0 and 2.0-2.2, respectively. These ratios were below the expected value for all samples. The TRIzol method gave a higher quantity of extracted RNA. The extraction with PowerLyzer gave also higher RNA amount.

To determine the efficiency of the extractions, a PCR was performed targeting As-p18 cDNAs. The pCr was done with the As-p18 primer pair.

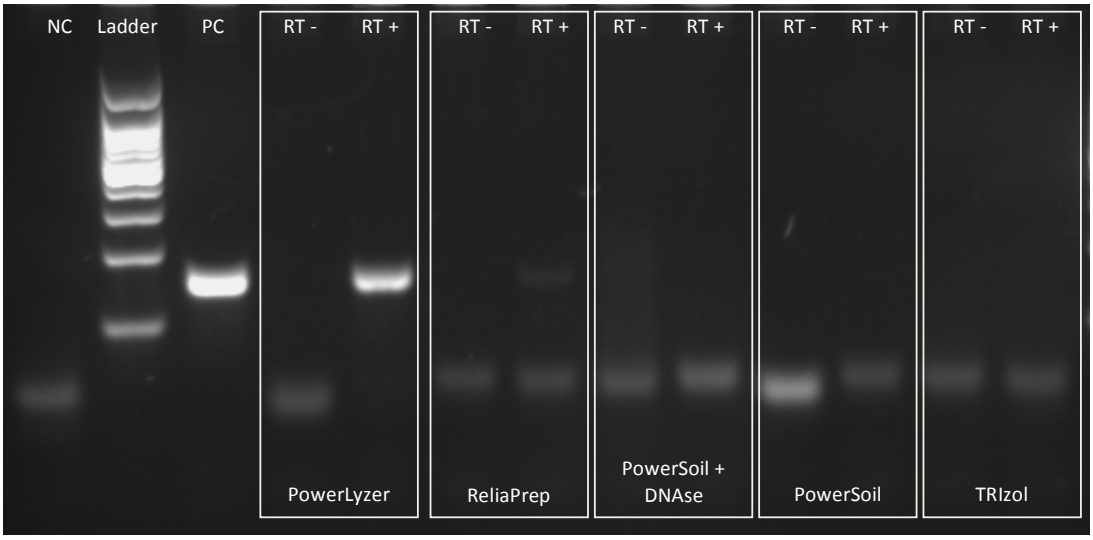



Figure 16: PCR products of the cDNAs from *Ascaris* eggs within sludge, extracted with five different methods

The result shows an intense fluorescence with the sample extracted with the kit PowerLyzer from Mobio and a less fluorescent signal with the sample extracted with the kit ReliaPrep from Promega.

A qPCR was done with all the samples, with dilutions: 1x, 5x, 10x, 20x and 40x. The table 20 summarizes the results obtained with the qPCR analysis.

Table 19: Quantification of the copy number of diluted As-p18 cDNA extracted from *Ascaris* eggs within sludge - the values obtained with diluted samples have already been corrected by the dilution factor

| Dilution | Number of copies [copies/µl] | | | |
|---|---|---|---|---|
| | PowerLyzer | ReliaPrep | PowerSoil | TRIzol |
| 1x | 31106 | n.d. | n.d. | n.d. |
| 5x | 27295 | n.d. | n.d. | n.d. |
| 10x | 63290 | 230 | n.d. | n.d. |
| 20x | 60600 | 400 | n.d. | 220 |
| 40x | n.d. | 320 | n.d. | n.d. |
| **Average** | **61945** | **317** | **n.d.** | **220** |

The qPCR confirmed the results obtained with the PCR analysis. The extraction made with the PowerLyzer kit gave more copy number than the other kits. A dilution of 10x or 20x seemed to be sufficient to eliminate the inhibition effect of the matrix. No signal was detected with a dilution of 40x.

## 4.3 Optimization of the reverse transcription methods

### *4.3.1 Reverse transcription of As-p18 mRNAs*

RT is an important step for the analysis of the expression level of RNAs of interest. In this chapter, the best RT methods for the analysis of the expression of As-p18 in *Ascaris* eggs is assessed. To this end, the RNA obtained from egg suspension using the ReliaPrepkit method was reverse transcribed in two different RT protocols.

The first tested method was the classical RT using anchored oligo-dT primer and the second is the kit BioScript All-in-one cDNA synthesis supermix. The agarose gel below shows the size of PCR products obtained with the RT products. The PCR was done with the As-p18 primer pair.

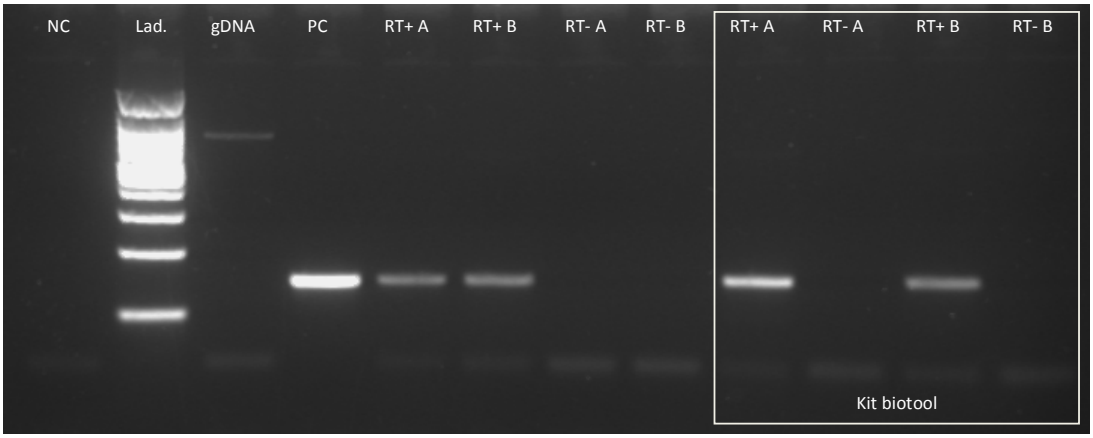



Figure 17: Comparison of the PCR products on As-p18 cDNAs obtained with two different RT methods

The well containing the gDNA was used to assess the purity of the RNA samples. The gel shows that the samples are not contaminated with gDNA.

The results of the RT(+) wells showed that both RT methods are efficient to transcribe As-p18 mRNAs.

### *4.3.2 Reverse transcription of CDP6 mRNAs*

To asses the efficiency of the RT methods on CDP6 mRNA, a suspension of eggs was incubated at 70°C for 7 days in order to induce the expression of CDP6. According to the results obtained with the extraction of As-p18 mRNA, the kit ReliaPrep was used to extract the total RNA of the sample. The amount of extracted RNA is 6.23 ng/µl with A260/A280 ratio of 1.92 and A260/A230 ratio of 1.22.

The classical RT using a specific primer or an anchored oligo-dT and the kit were tested. The cDNAs were analyzed by PCR, using the As-CDP6-x7 primer pair. The results of the PCR are shown in the figure 18.

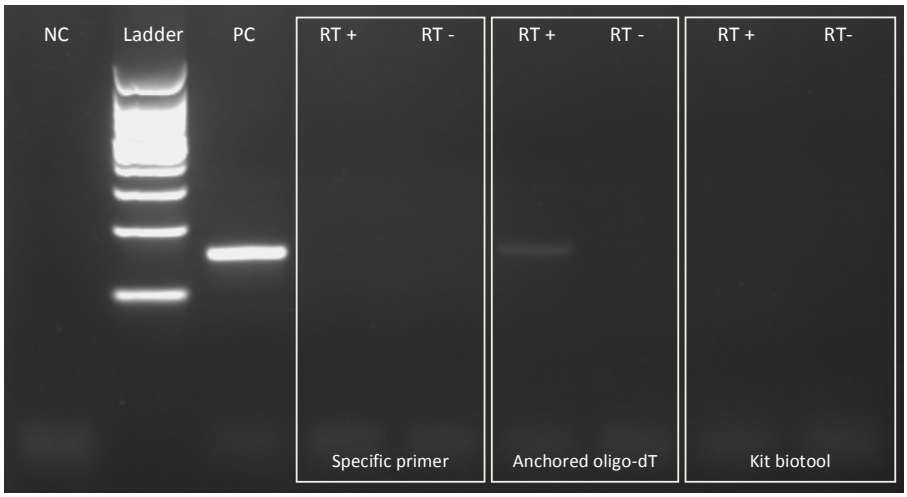



Figure 18: Comparison of the PCR products on CDP6 cDNAs obtained with three different RT methods

The classical RT method using anchored oligo-dT primer gives a positive result in the agarose gel, while with the other methods no signal is detected.
A qPCR was done with the cDNAs obtained with the classical RT method using anchored oligo-dT. The sample was diluted to reduce the effects of PCR inhibitors that might be present. There was no result.

## 4.4 Primer pair evaluation

### *4.4.1 Primers targeting the development marker: As-p18*

In order to compare the efficiency of the primer pairs designed to detect and quantify As-p18 cDNAs, a second PCR was done with cDNAs transcribed from the RNA extracted with the kit ReliaPrep™ RNA Cell Miniprep System. This time, the primers pair targeting the exon 2 of the cDNA was used (As-p18-x2 primer pair). The RT was done with the biotool kit method. The agarose gel in figure 19 shows the result of the PCR.

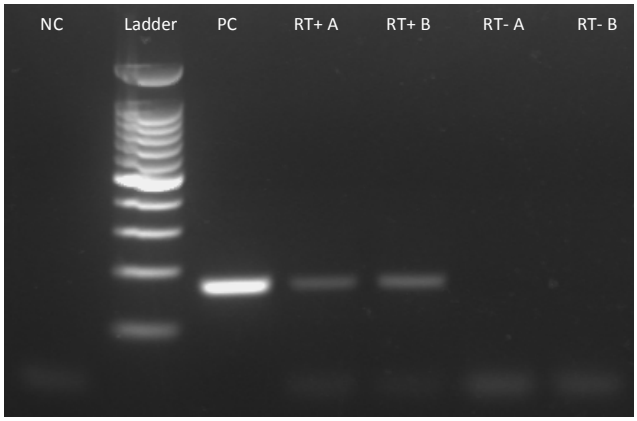



Figure 19: PCR on As-p18 cDNAs using AS-p18-x2 primer pair

As expected, the RT(+) samples had the right signal for both samples A and B while there was no signal with the RT(-) samples. In comparison with the signals obtained with the other primers, the signals obtained with these ones were less strong.

The cDNAs transcribed by the biotool kit from sample A were also analyzed by qPCR using both primer pairs. The results are summarized in table 21.

Table 20: Comparison of the qPCR quantification of As-p18 using two different primer pairs

| | | **As-p18 primer pair** | **As-p18-x2 primer pair** |
|---|---|---|---|
| Reaction efficiency | | 0.86 | 0.84 |
| Copies of As-p18/µl of sample | | 8321 | 3693 |
| Variances between the given concentration of plasmids and the calculated concentration | Minimum | 2.4 % | 4.9 % |
| | Maximum | 31.3 % | 38.3 % |
| | Over 20% | 3 | 4 |

The difference between the efficiencies using As-p18 or As-p18-x2 primers is not significant. However, the number of copies of As-p18 cDNAs obtained with As-p18 primer pair is more than twice the number obtained with As-p18-x2.
The variances between the given concentration of plasmids and the calculated concentration are higher when using the As-p18-x2 primer pair.

### 4.4.2 *Primers targeting the apoptosis marker: CDP6*

In order to compare the efficiency of the primer pairs designed to detect and quantify CDP6 cDNAs, a second PCR was done with cDNAs analyzed in chapter 4.3.2 using the As-CDP6 primer pair. The samples of gDNA extracted with the treatment L and J were also analyzed by PCR using the As-CDP6 primer pair. The results are shown in the agarose gel below.

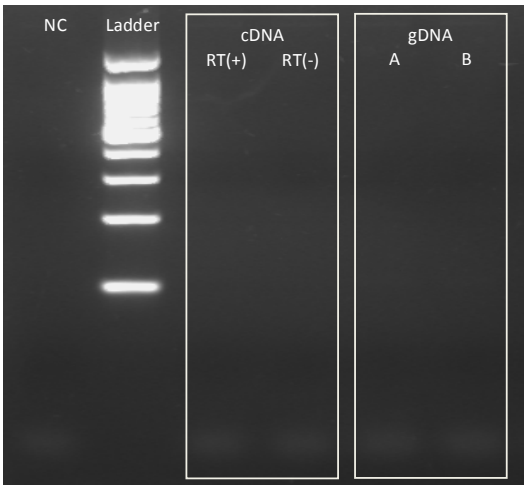



Figure 20: PCR on CDP6 cDNAs using AS-CDP6 primer pair

The expected size of the PCR product of the cDNA is 150 bp, while it is about 1450 bp for gDNA. Yet, whatever the template (cDNA or gDNA), no fluorescence signal is detected in the agarose gel.

## 4.5 Development of the quantification method

### 4.5.1 *Standard development for CDP6-x7*

The standard used for the quantification targeting CDP6 was done with a DNA extract. A PCR targeting the exon 7 of the coding gene of CDP6 was done. An agarose gel was done in order to confirm that the target was amplified.

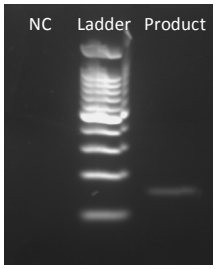



Figure 21: PCR results on gDNA sample targeting CDP6 with As-CDP6-x7 primer pair

The PCR product was purified and A-tailed. Then, a ligation step was done with the pGEM-T Easy system. A culture of competent *E.coli* was transformed with the pGEM-T-CDP6-x7 plasmids. The transformed cells were cultivated in a Petri dish with LB and ampicillin. 15 colonies were picked up and re-spread in another Petri dish thanks to a toothpick. The toothpick was then cleaned in a PCR tube containing water. The total DNA present in the PCR tubes were extracted and a PCR targeting the region between the region T7 and the region SP6 of the plasmid was done for each tube. Without the insert, the product is about 150 bp and with the insert, it is about 300 bp. The agarose gel in figure22 shows the results of the PCR done with each colony.

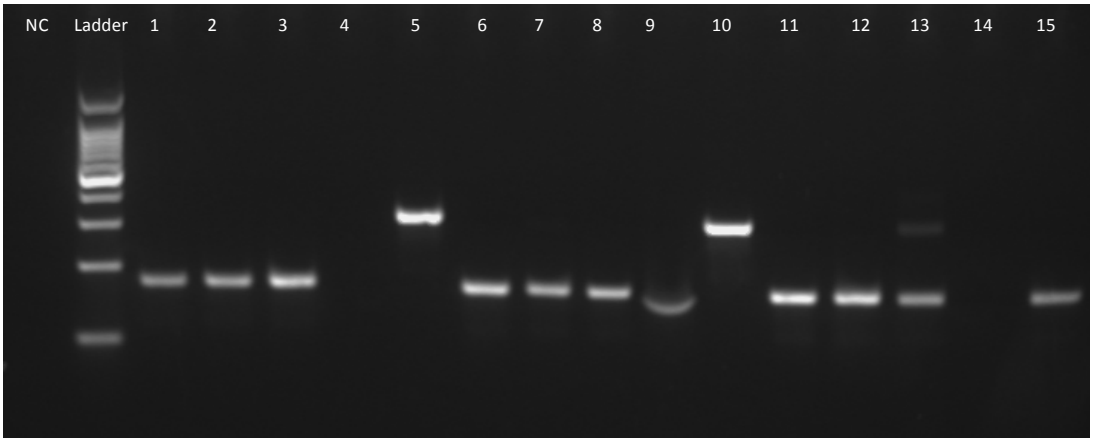



Figure 22: Colony PCR results on the cloned plasmids from 15 different colonies

As shown in the agarose gel, two among the 15 picked colonies have the ligated plasmid.

For the development of the standard, the colonies 5 and 10 were cultivated overnight at 37°C in a LB broth. Then, the plasmids of these colonies were extracted. The inserts were sequenced and the results were compared to the sequence of the amplicon obtained with the As-CDP6-x7 primer pair. The results of the sequencing showed that the sequence of the insert in colony 10 has only 96% of similarities with the expected sequence, while the insert in colon5 has 100% of similarities. The alignment of the sequences is presented in appendix 14.

Thus, the plasmids obtained from colony 5 were diluted to a final concentration of 1 ng/µl and analyzed by PCR with the As-CDP6-x7 primer pair. The agarose gel below shows the result of the analysis.

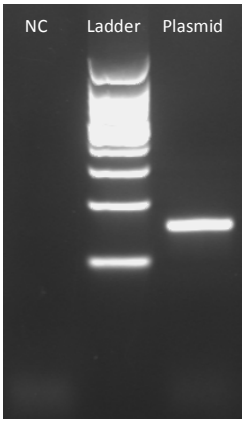



Figure 23: PCR on the selected plasmid targeting CDP6

For the rest of the project, these plasmids were used as a positive control for the detection of CDP6 and as a standard for the qPCR targeting CDP6.

### *4.5.2 Standard curves for the qPCR methods*

For each target, a standard curve was set up from a standard linearized plasmid. The quantifications of these standards allowed calculating the number of copies of the target per µl of sample. The calculation of the number of copies of each target is summarized in table 22.

Table 21: Calculation of the copy numbers of each target in their respective plasmid standard

| Target | Target size | Plasmid size | MW | Conc. | Copy number in original sample | Dilution series |
|---|---|---|---|---|---|---|
| | [bp] | [bp] | [g/mol] | [ng/µl] | [copies/µl] | |
| ITS-1 | 82 | 4130 | 2684500 | 14.5 | $3.26 \times 10^{9}$ | $10^{-1}$ to $10^{-9}$ |
| As-p18 | 150 | 3953 | 2569450 | 7.2 | $1.69 \times 10^{9}$ | $10^{-1}$ to $10^{-9}$ |
| CDP6 | 155 | 3170 | 2060500 | 10.2 | $2.98 \times 10^{9}$ | $10^{-1}$ to $10^{-9}$ |

### *4.5.3 Characterization of the qPCR methods*

In order to validate the quantification method, the standard curve was made in triplicate for each target. The LOD and LOQ were determined and are shown in the table below. The qPCR results are available in appendixes 16 to 18.

Table 22: Characterization of the quantification methods (linearity range and efficiency)

| | ITS-1 | As-p18 | CDP6 |
|---|---|---|---|
| LOD | $3.26 \times 10^{1}$ | $1.69 \times 10^{1}$ | $2.98 \times 10^{0}$ |
| LLOQ | $3.26 \times 10^{1}$ | $1.69 \times 10^{1}$ | $2.98 \times 10^{0}$ |
| ULOQ | $3.26 \times 10^{9}$ | $1.69 \times 10^{9}$ | $2.98 \times 10^{9}$ |
| Efficiency | 0.81 | 0.81 | 1.03 |

It is important to notice that the ULOQ calculated here is the maximum amount tested during the project. The results show that the reaction efficiencies of each method are on the values range of an ideal PCR reaction (1.0 ± 0.2).

# 5. Discussion

## 5.1 Optimization of the *Ascaris* eggshell breaking method

The treatment by bleaching does not seem to improve the extraction. On contrary, this treatment even gave the lowest amount of extracted DNA (1.7 ng/µl). As this treatment involves several steps of washing, the freed cells and the unbroken eggs may have been discarded with the supernatant during one (or more) of these washing steps. It is interesting to notice that for the sample treated by bleaching, the dilutions did not increase the signal in the agarose gel. Thus, the bleaching treatment could help to minimize the presence of inhibitors for the PCR.

The highest amount of extracted DNA was achieved with methods combining enzymatic and mechanical treatments. Even when not combined with enzymatic treatment, the mechanical treatment gave high amount of extracted DNA. Thus, mechanical pressure is the best way to break *Ascaris* eggshells. Three different mechanical treatments were tested: ultrasonication, mortar crushing and bead beating.
It was noticed that the amount of extracted DNA increases proportionally to the duration of ultrasonication. This trend is also observed with the qPCR results. The morphological changes observed by light microscopy showed that the eggshells were broken after ultrasonication and mortar crushing. Ultrasonication causes a kind of fissure in the eggshell, thus inducing the release of the cells inside eggs. An observation by electronic microscope was done by Loreille et al. [26] after passing the *Ascaris* eggs through ultrasonic wave at 60°C for 20 minutes. In the reported image, it can be clearly observed that the eggshell presented a similar fissure.
The crushing also causes a fissure in the shell. Interestingly, the shell is not completely broken but present one exit point for the cells or larva. This point can be the region corresponding to the point where the hatching of the larva happens. It is called operculum and represents the single point of weakness of the eggshell [5].
Bead beating is the harder mechanical treatment. Four of the thirteen tested treatments had in common that they involved a bead-beating step. Except when it was combined with bleaching washing and suspension in EDTA/sarcosyl, the extraction gave a DNA amount higher than 20 ng/µl, which is a good value compared to the results of those extracted without mechanical treatment. The suspension in EDTA/sarcosyl induced the formation of foam during the bead beating. It can explain the low amount of DNA obtained with this treatment.

For the rest of the experiments, the mortar treatment was chosen as pre-treatment for nucleic acid extraction. For further improvements of eggshell breaking method, bead beating should be more exploited. Indeed, mortar crushing is a manual treatment and thus presents many fluctuations in term of strength and pressure. In addition, the microscopy photographs have shown that the cells were not totally released. On the other hand, bead beating is an automated processing and thus could decrease the risk of fluctuations in results.

<u>Evaluation of the absorbance measurement of DNA molecules</u>
The huge differences observed between the quantification value with NanoDrop and Qubit systems are probably due to the low DNA concentration in the samples and the lower sensitivity of the NanoDrop system compared to the Qubit system. Indeed, although NanoDrop system is not recommended for concentrations below 2 ng/µl, the precision of the method is questionable even below 10 ng/µl [27]. By contrast, the sensitivity range of the Qubit system is from 10 pg/µl to 1 µg/µl. But the advantage of NanoDrop is that the A260/A280 and A260/A230 ratios can give some indications about the purity of the sample, which is not possible with the Qubit system.
For further studies on the molecular analysis of *Ascaris* eggs, the Qubit HS system should be used systematically to quantify the extracted DNA and RNA.

Note that the low concentration of DNAs can be due to the extraction method itself. Indeed, the extraction method using Maxwell 16 robot elutes the DNAs in 300 µl of elution buffer. It would be possible to increase the concentration of DNAs by precipitating the samples and by resuspending them in a lower volume of $ddH_2O$. Of course, some precautions have to be taken to minimize the losses of materials and the contaminations.

## 5.2 Optimization of the RNA extraction

Concerning the RNA extraction from egg suspension, there is no significant difference of extraction yield among the three tested methods (Reliaprep RNA Cell/Tissue Miniprep system and TRIzol-based method).

The PCR results, targeting As-p18, show that the samples extracted with the kits have more fluorescence than the sample extracted with the TRIzol-based method. The interpretation of this result should however take into account the fact that contaminations were observed in the negative control and the RT(-) samples. As there is no signal around 750 bp, these contaminations are probably not a DNA contamination. It may come from a contamination from the positive control during the preparation of the reaction mix for the PCR. Thus, the signal obtained in the RT(+) of the TRIzol sample is not considered as a result of the PCR on the cDNAs but as a result of the contamination.
In the TRIzol-based method, the RNAs are extracted and purified in a column. Then, they are eluted from the column before DNAs treatment. This step could induce the loss of RNAs by contamination with RNAse. Indeed, the samples are treated outside the hood because the DNAse reaction takes place at 37°C and there is no heater inside the hood. By contrast, samples extracted with the kits are treated on-column at room temperature by DNAse. Thus, these samples stay inside the hood and the risk of contamination with RNAse is minimized.

Concerning the RNA extraction from eggs within sludge, the TRIzol-based method also gave no result on the agarose gel.
A sample having a ratio near to 2.0 is considered as pure. Thus, according to this ratio, the purest sample is the one extracted with the PowerLyzer RNA isolation kit, with a ration value of 1.97. The amount of RNA obtained with this kit is also significant (16.8 ng/µl) compared to the other kits (about 6 ng/µl).
By contrast, all the samples have a low A260/A230 ratio. The expected value for pure samples is between 2.0 and 2.2, and the extracted samples have a ratio value between 0.5 and 1.2. This low value indicates that the sample is not as pure as expected. But, this value is also influenced by the interference of residual phenol or other reagent used during the extraction on the absorbance measurement.

The PCR results show a significant signal in the RT(+) of the sample extracted with the PowerLyzer RNA isolation kit and a slight signal in the RT(+) of the sample extracted with the Reliaprep RNA Miniprep system. The PowerLyzer method has a bead-beating step in its protocol. As discussed before, bead beating could be an efficient way to break eggshells and this result confirm this theory, even within a complex matrix.

## 5.3 Optimization of the RT method

Both classical RT method and RT kit method give a satisfactory response with PCR analysis on As-p18 mRNA. The RT kit seems to be a good choice for the continuation of the project. Indeed, it is timesaving and the risk of contamination or loss of materials is minimized, as there is less product handling.
But, in the case of the CDP6 mRNA analysis, the sample reverse transcribed by the biotool kit method did not give any positive result. The RT using anchored oligo-dT is the only method showing a positive signal above the three tested methods (classical with specific primer, anchored oligo-dT and kit method). It is important to notice that the amount of RNA was too low for the recommended amount when using the kit. In addition, the kit uses a "standard" oligo-dT primer and not an "anchored" one. It means that the primers can bind anywhere in the poly-A regions of the mRNAs and the resulting cDNA is truncated.

## 5.4 Primer pair selection

- Development marker: As-p18

According to the PCR results, detection of As-p18 mRNA is possible with both As-p18 and As-p18-x2 primer pairs. The advantage of the As-p18 primer pair is the possibility to differentiate between cDNAs and gDNAs. With this primer pair, the size of the amplicon of cDNAs is 151 bp while the size of the amplicon of gDNAs is about 1000 bp (cf. Figure 15).
As regards the qPCR results, the quantification using As-p18 primer pair gives an amount more than twice the number obtained when using As-p18-x2 primer pair. The standard values are more accurate with the As-p18 primer pair.
In the light of these results, it was assumed that As-p18 primer pair is the most appropriate for the continuation of the project.

- Apoptosis marker: CDP6

A PCR on CDP6 cDNA and gDNA using the As-CDP6 primer pair gives no amplification (cf. Figure 20). When using As-CDP6-x7 primer pair, amplifications were detected whatever the sample is (cf. Figure 18 and Figure 21). This is why the As-CDP6-x7 primer pair was chosen for the project.
The fact that there were no results in qPCR can be due to the parameters set on the qPCR. Indeed, the Cts of the samples were lower than 1.27 x $Ct_{NTC}$ and the program considered the signals as a noise. It is certainly because of the low amount of cDNAs. Indeed, the incubation time of the *Ascaris* eggs at 70°C was 7 days. CDP6 should be expressed when cells begin to die. It is possible that in 7 days, the majority of the cells are already dead.

## 5.5 Evaluation of the qPCR method

- Characterization of the methods

The qPCR methods targeting ITS-1 rDNA and As-p18 cDNA have a relatively high LLOQ, respectively 33 copies/µl and 17 copies/µl. The LLOQ of the method targeting CDP6 cDNA is low enough (3 copies /µl). As only ten-fold serial dilutions were done, assessing the detection and quantification with supplementary dilutions could refine these results.
High LLOQ means that the method is not sensitive enough to quantify a low amount of DNA. The sensitivity can be improved by testing different annealing temperatures or by increasing the number of cycles during the PCR.

- Inhibition evaluation

More dilution is required for samples from eggs within sludge than for samples from purified eggs suspension (cf. Table 16 and Table 19). Indeed, it was shown that within sludge, there are typical PCR inhibitors, such as complex polysaccharides, bile salts, urate and lipids [28]. Polysaccharides are a problem because they can change the chemical properties of DNAs and RNAs. The ability of the salts (bile salts and urate) to degrade or sequestrate nucleic acids is another big issue.
In order to reduce the amount of PCR inhibitors in the sample, some articles propose an additional step when extracting nucleic acids. It was proposed to heat the sample before the PCR or to treat the sample with activated carbon or by chromatography [28–31].

Concerning the quantification itself, the results present many fluctuations for the same sample and in the same qPCR run. It could be interesting to use an internal standard. Indeed, internal standard couls make the results more precise and help to detect low DNA concentrations.

## 6. Conclusion and outlook

The first objective of the project was to optimize the nucleic acid extraction, especially concerning the eggshell-breaking step. Mechanical treatments are the most efficient to break the eggshells and extract the nucleic acids. Thus, in this project, the retained steps of the method were:

- a mortar-crushing step prior to DNA or RNA extraction
- RNA extraction by the ReliaPrep™ RNA Cell/Tissue Miniprep System for *Ascaris* egg suspension and by the PowerLyzer UltraClean Tissue and Cells RNA isolation kit for *Ascaris* eggs within sludge
- a classical RT using an anchored oligo-dT

The method has still to be refined. Indeed, the fluctuations on the eggshell breaking method could be limited by choosing bead beating treatment in spite of mortar crushing. It is also better to use the PowerLyzer UltraClean Tissue and Cells RNA isolation kit for pure *Ascaris* eggs as well as for eggs within sludge.

The second objective of the project was to design and optimize the molecular analysis of the target sequences. For the molecular analysis, the primer pair targeting two different exons in the As-p18 cDNA was selected for the assessment of the As-p18 expression level. The primer pair targeting a unique exon (exon 7) in the CDP6 cDNA was selected for the assessment of the CDP6 expression level in *Ascaris suum* eggs. The conditions of the qPCR methods have not been assessed enough to establish a final method.
To optimize the qPCR method, the addition of internal standard could be a great advantage and the qPCR conditions have to be evaluated.

The issue of the eggshell strength is resolved. But, additional experiments are needed to validate the method and set up the quantification method. It would be helpful to assess the expression level of As-p18 and CDP6 in *Ascaris* eggs by incubating them in the right conditions. A comparison with a microscopic enumeration could validate the robustness of the method.

A method that might be interesting is the ImmunoPCR, a PCR targeting proteins. The method works like an ELISA test: the protein of interest is captured by a specific antibody bound with a detection antibody. The difference lies in the way of detection: in ELISA, the detection antibody is bound to an enzyme that converts a substrate into a chromophore product, while in the iPCR, it is bound to a DNA molecule that is quantified by qPCR.

## Acknowledgements

I would like to thank Prof. Sergio Schmid. He gave me the opportunity to do my bachelor project outside the HEI. He also gave me precious advice before I started the project.
I also express my thanks to Prof. Christof Holliger for allowing me to join his team. I really appreciate that he gave me the opportunity to handle such a big challenge and discover the LBE.

I would like to express my gratitude to my supervisor Julien Maillard for his help and support. He supervised me throughout the project and helped me to look positively at my results even when it was not what I expected. He showed me "where to find what" in the laboratory and he took time to guide me.

Furthermore, I would like to thank Magalie Bassan who helped me to focus on the essential issue and on the aim of the project.
And, of course, I do not forget all the "*Ascaris* team": thanks to Léa Randin for her presence and help.

I would like to thank all the laboratory team who welcomed me. I would like to give a special thanks to Geraldine Buttet for helping me to sequence my inserts and for her so clear and detailed protocols.

I want to thank wholeheartedly my husband, Nicolas Estoppey, for his support, prayers and help. I thank him for his constant motivating attitude and helpful advices. He gave me the external view that I needed to put pieces together.

I also thank my lovely family and friends who supported me by their prayers and encouraging words, even when they did not understand what I was talking about.

## Bibliography


[1] World Health Organization, Excreta and greywater use in agriculture, 2006.

[2] C. Dold, C. V Holland, Helminth-Nematode: Ascaris, in: Encycl. Food Saf., 2014: pp. 83–89. doi:10.1016/B978-0-12-378612-8.00141-4.

[3] V.T. Yen-phi, A. Rechenburg, B. Vinneras, J. Clemens, T. Kistemann, Science of the Total Environment Pathogens in septage in Vietnam, Sci. Total Environ. 408 (2010) 2050–2053. doi:10.1016/j.scitotenv.2010.01.030.

[4] A.F. Gabrielli, A. Montresor, L. Savioli, Soil-transmitted Helminthiasis, in: F. Bruschi (Ed.), Helminth Infect. Their Impact Glob. Public Heal., Springer, 2014: pp. 275–297. doi:10.1007/978-3-7091-1782-8.

[5] D.A. Wharton, The production and functional morphology of helminth egg-shells, Parasitology. 86 (2009) 85. doi:10.1017/S003118200005085X.

[6] R.G. Feachem, D.J. Bradley, H. Garelick, D.D. Mara, Sanitation and disease: health aspects of excreta and wastewater management., 1983. doi:10.1016/0035-9203(84)90011-7.

[7] B.M. Pecson, J.A. Barrios, D.R. Johnson, K.L. Nelson, A real-time PCR method for quantifying viable Ascaris eggs using the first internally transcribed spacer region of ribosomal DNA, Appl. Environ. Microbiol. 72 (2006) 7864–7872. doi:10.1128/AEM.01983-06.

[8] M. Raynal, E.N. Villegas, K.L. Nelson, Enumeration of viable and non-viable larvated Ascaris eggs with quantitative PCR, J. Water Health. 10 (2012) 594–604. doi:10.2166/wh.2012.101.

[9] A.R. Jex, S. Liu, B. Li, N.D. Young, R.S. Hall, Y. Li, et al., Ascaris suum draft genome, (2011). doi:10.1038/nature10553.

[10] J. Wang, J. Garrey, R.E. Davis, Transcription in pronuclei and one- to four-cell embryos drives early development in a nematode, Curr. Biol. 24 (2014) 124–133. doi:10.1016/j.cub.2013.11.045.

[11] P. Gaspard, J. Wiart, J. Schwartzbrod, A Method for Assessing the Viability of Nematode Eggs in Sludge, Environ. Technol. 17 (1996) 415–420. doi:10.1080/09593331708616401.

[12] M. Szymański, T. Opioła, M.Z. Barciszewska, J. Barciszewski, The nucleotide sequence of 5S ribosomal RNA from the parasitic nematode Ascaris suum. Evolutionary relationships in nematodes, Biochim. Biophys. Acta - Gene Struct. Expr. 1308 (1996) 251–255. doi:10.1016/0167-4781(96)00115-7.

[13] A. Karkashan, B. Khallaf, J. Morris, N. Thurbon, D. Rouch, S.R. Smith, et al., Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy, the BacLight Live/Dead viability assay and other vital dyes, Water Res. 68 (2015) 533–544. doi:10.1016/j.watres.2014.10.003.

[14] D. Leles, A. Araújo, A.C.P. Vicente, A.M. Iñiguez, Molecular diagnosis of ascariasis from human feces and description of a new Ascaris sp. genotype in Brazil, Vet. Parasitol. 163 (2009) 167–170. doi:10.1016/j.vetpar.2009.03.050.

[15] C.A. Gordon, D.J. Gray, G.N. Gobert, D.P. McManus, DNA amplification approaches for the diagnosis of key parasitic helminth infections of humans, Mol. Cell. Probes. 25 (2011) 143–152. doi:10.1016/j.mcp.2011.05.002.

[16] B. Mei, M.W. Kennedy, J. Beauchamp, P.R. Komuniecki, R. Komuniecki, Secretion of a novel, developmentally regulated fatty acid-binding protein into the perivitelline fluid of the parasitic nematode, Ascaris suum, J. Biol. Chem. 272 (1997) 9933–9941. doi:10.1074/jbc.272.15.9933.

[17] H.P. Su, K. Nakada-Tsukui, A.C. Tosello-Trampont, Y. Li, G. Bu, P.M. Henson, et al., Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP), J. Biol. Chem. 277 (2002) 11772–11779. doi:10.1074/jbc.M109336200.

[18] Z. Zhou, E. Hartwieg, H.R. Horvitz, CED-1 is a transmembrane receptor that mediates cell corpse engulfment in C. elegans, Cell. 104 (2001) 43–56. doi:10.1016/S0092-8674(01)00190-8.

[19] Q. a. Liu, M.O. Hengartner, Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in C. elegans, Cell. 93 (1998) 961–972. doi:10.1016/S0092-8674(00)81202-7.

[20] Y.-M. Yu, Y.-H. Cho, Y.-N. Youn, J.H. Quan, I.-W. Choi, Y.-H. Lee, Quantitative evaluation of viability- and apoptosis-related genes in Ascaris suum eggs under different culture-temperature conditions., Korean J. Parasitol. 50 (2012) 243–7. doi:10.3347/kjp.2012.50.3.243.

[21] O. Loreille, E. Roumat, O. Verneau, F. Bouchet, C. Hänni, Ancient DNA from Ascaris: Extraction amplification and sequences from eggs collected in coprolites, Int. J. Parasitol. 31 (2001) 1101–1106. doi:10.1016/S0020-7519(01)00214-4.

[22] A. Spicher, A Method for RNA Isolation from Ascaris Eggs and Embryos, Worm Breeder's Gaz. 12(3) 15. (1992).

[23] L.O. Andersen, D. Röser, P. Nejsum, H.V. Nielsen, C.R. Stensvold, Is supplementary bead beating for DNA extraction from nematode eggs by use of the NucliSENS easyMag protocol necessary?, J. Clin. Microbiol. 51 (2013) 1345–7. doi:10.1128/JCM.03353-12.

[24] Polymerase chain reaction, (n.d.). https://commons.wikimedia.org/wiki/File:Polymerase_chain_reaction.svg (accessed July 7, 2015).

[25] Promega, pGEM-T and pGEM-T Easy Vector Systems Technical Manual, 2009.

[26] O. Loreille, F. Bouchet, Evolution of Ascariasis in Humans and Pigs: A Multi-disciplinary Approach, Mem. Inst. Oswaldo Cruz. 98 (2003) 39–46. doi:10.1590/S0074-02762003000900008.

[27] Qubit® Fluorometer vs. Competitors, (n.d.). http://www.lifetechnologies.com/ch/en/home/life-science/laboratory-instruments/fluorometers/qubit/qubit-fluorometer/qubit-vs-competitors.html (accessed August 5, 2015).

[28] C. Schrader, A. Schielke, L. Ellerbroek, R. Johne, PCR inhibitors - occurrence, properties and removal, J. Appl. Microbiol. 113 (2012) 1014–1026. doi:10.1111/j.1365-2672.2012.05384.x.

[29] W. Abu Al-Soud, P. Rådström, Capacity of nine thermostable DNA polymerases To mediate DNA amplification in the presence of PCR-inhibiting samples., Appl. Environ. Microbiol. 64 (1998) 3748–53. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=106538&tool=pmcentrez&rendertype=abstract (accessed June 6, 2015).

[30] U. Chaturvedi, A.K. Tiwari, B. Ratta, P. V Ravindra, Y.S. Rajawat, S.K. Palia, et al., Detection of canine adenoviral infections in urine and faeces by the polymerase chain reaction., J. Virol. Methods. 149 (2008) 260–3. doi:10.1016/j.jviromet.2008.01.024.

[31] A. Scipioni, A. Mauroy, D. Ziant, C. Saegerman, E. Thiry, A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition., Virol. J. 5 (2008) 94. doi:10.1186/1743-422X-5-94.

[32] NCBI GenBank database, (n.d.). http://www.ncbi.nlm.nih.gov (accessed April 17, 2015).

[33] Basic Principles of RT-qPCR, (n.d.). https://www.lifetechnologies.com/ch/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html (accessed July 7, 2015).

## Appendixes

Appendix 1 : Ascaris lumbricoides ITS-1 rDNA sequence [32] ..... 47
Appendix 2 : Ascaris suum ITS-1 rDNA sequence [32] ..... 48
Appendix 3 : As-p18 cDNA sequence [32] ..... 49
Appendix 4 : As-p18 gDNA coding sequence [32] ..... 50
Appendix 5 : Ascaris suum CDP6 gDNA coding sequence [32] ..... 52
Appendix 6: RNA extraction protocol of LBE ..... 56
Appendix 7: Reliaprep™ RNA Tissue Miniprep System quick protocol ..... 58
Appendix 8: Reliaprep™ RNA Cell Miniprep System quick protocol ..... 60
Appendix 9: BioScript All-in-One cDNA Synthesis SuperMix protocol ..... 62
Appendix 10: PowerLyzer® UltraClean® Tissue and Cells RNA isolation kit ..... 64
Appendix 11: RNA PowerSoil total RNA isolation kit ..... 67
Appendix 12: QIAprep® Spin Miniprep kit protocol ..... 70
Appendix 13: pGEM-T system protocol ..... 72
Appendix 14: QIAquick PCR Purification kit protocol ..... 74
Appendix 15: Sequences alignement of the inserts CDP6-x7 in pGEM-T Easy ..... 76
Appendix 16: qPCR results for the characterization of pASI standard curve ..... 77
Appendix 17: qPCR results for the characterization of pASP standard curve ..... 79
Appendix 18: qPCR results for the characterization of pASC standard curve ..... 81

## ***Appendix 1 : Ascaris lumbricoides*** **ITS-1 rDNA sequence** [32]

***Ascaris lumbricoides*** **first internal transcribed spacer ribosomal DNA**

LOCUS AJ000895 446 bp DNA linear INV 15-MAR-2000
DEFINITION Ascaris lumbricoides first internal transcribed spacer ribosomal DNA.
ACCESSION AJ000895
VERSION AJ000895.1 GI:6850909
KEYWORDS ITS1.
SOURCE Ascaris lumbricoides (common roundworm)
ORGANISM Ascaris lumbricoides
Eukaryota; Metazoa; Ecdysozoa; Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae; Ascaris.
REFERENCE 1
AUTHORS Zhu,X., Chilton,N.B., Jacobs,D.E., Boes,J. and Gasser,R.B.
TITLE Characterisation of Ascaris from human and pig hosts by nuclear ribosomal DNA sequences
JOURNAL Int. J. Parasitol. 29 (3), 469-478 (1999)
PUBMED 10333331
REFERENCE 2 (bases 1 to 446)
AUTHORS Zhu,X.
TITLE Direct Submission
JOURNAL Submitted (30-JAN-1998) Zhu X., Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, AUSTRALIA
FEATURES Location/Qualifiers
source 1..446
/organism="Ascaris lumbricoides"
/mol_type="genomic DNA"
/isolate="Alum1, Alum3-Alum7"
/db_xref="taxon:6252"
/dev_stage="Adult"
gene 1..446
/gene="First internal transcribed spacer ribosomal DNA"
misc_feature 1..446
/gene="First internal transcribed spacer ribosomal DNA"
ORIGIN
1 atcgagcaga aaaaaaaaag tctccgaacg tgcacataag tactatttgc gcgtatacgt
61 gagccacata gtaaattgca cacaaatgtg gtgatgtaat agcagtcggc ggtttctttt
121 tttttggcgg acaattgcat gcgatttgct atgtgttgag ggagaatagg tggcatgttg
181 ggcttgttag aaaggcatgc stagcgctta ttttcccgct atttcgtaac aacggtgtcc
241 attttggcgt ctacgcttca ccgagctatc gcctggaccg tcggtagcga tgaaaggtgg
301 agagaaagct cctcgtttcg rgtcgagtag actcaatgag cctcagcttg gaggccgcca
361 aaactcaaaa aacacaatca cttttgaaaa tctattctaa tgaaagatgc taaattttgt
421 ttagtatctt cgaattgtaa gatgaa
//

## ***Appendix 2 : Ascaris suum*** **ITS-1 rDNA sequence** [32]

***Ascaris suum*** **first internal transcribed spacer ribosomal DNA**

```
LOCUS       AJ000896               450 bp    DNA     linear   INV 15-MAR-2000
DEFINITION  Ascaris suum first internal transcribed spacer ribosomal DNA.
ACCESSION   AJ000896
VERSION     AJ000896.1  GI:6850910
KEYWORDS    ITS1.
SOURCE      Ascaris suum (pig roundworm)
  ORGANISM  Ascaris suum
            Eukaryota; Metazoa; Ecdysozoa; Nematoda; Chromadorea; Ascaridida;
            Ascaridoidea; Ascarididae; Ascaris.
REFERENCE   1
  AUTHORS   Zhu,X., Chilton,N.B., Jacobs,D.E., Boes,J. and Gasser,R.B.
  TITLE     Characterisation of Ascaris from human and pig hosts by nuclear
            ribosomal DNA sequences
  JOURNAL   Int. J. Parasitol. 29 (3), 469-478 (1999)
   PUBMED   10333331
REFERENCE   2  (bases 1 to 450)
  AUTHORS   Zhu,X.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JAN-1998) Zhu X., Department of Veterinary Science,
            The University of Melbourne, 250 Princes Highway, Werribee,
            Victoria 3030, AUSTRALIA
FEATURES             Location/Qualifiers
     source          1..450
                     /organism="Ascaris suum"
                     /mol_type="genomic DNA"
                     /isolate="Asuu3, Asuu4G, AsuuPN3-AsuuPN5, Asuu5, Asuu7"
                     /db_xref="taxon:6253"
                     /dev_stage="Adult"
     gene            1..450
                     /gene="First internal transcribed spacer ribosomal DNA"
     misc_feature    1..450
                     /gene="First internal transcribed spacer ribosomal DNA"
ORIGIN
        1 atcgagcaga aaaaaaaraa agtctccgaa cgtgcacata agtactattt gcgcgtatac
       61 gtgagccaca tagtaaattg cacacaaatg tggtgatgta atagcagtcg gcggtttctt
      121 ttttttttg gccgacaatt gcatgcgatt tgctatgtgt tgagggagaa taggtggcat
      181 gttgggcttg ttagaaaggc atgcstagcg cttattttcc cgctatttcg taacaacggt
      241 gtccaatttg gcgtctacgc ttcaccgagc tatcgcctgg accgtcggta gcgatgaaag
      301 gtggagagaa agctcctcgt ttcgagtcga gtagactcaa tgagcctcag cttggaggcc
      361 gccaaaactc aaaaaacaca atcacttttg aaaatctatt ctaatgaaag atgctaaatt
      421 ttgtttagta tcttcgaatt gtaagatgaa
//
```

## ***Appendix 3 : As-p18 cDNA sequence*** [32]

***Ascaris suum* fatty acid binding protein homolog As-p18 mRNA, complete cds**

```
LOCUS       ASU51906               628 bp    mRNA    linear   INV 01-DEC-1999
DEFINITION  Ascaris suum fatty acid binding protein homolog As-p18 mRNA,
            complete cds.
ACCESSION   U51906
VERSION     U51906.1  GI:1272383
KEYWORDS    .
SOURCE      Ascaris suum (pig roundworm)
  ORGANISM  Ascaris suum
            Eukaryota; Metazoa; Ecdysozoa; Nematoda; Chromadorea; Ascaridida;
            Ascaridoidea; Ascarididae; Ascaris.
REFERENCE   1  (bases 1 to 628)
  AUTHORS   Mei,B., Kennedy,M.W., Beauchamp,J., Komuniecki,P.R. and
            Komuniecki,R.
  TITLE     Secretion of a novel, developmentally regulated fatty acid-binding
            protein into the perivitelline fluid of the parasitic nematode,
            Ascaris suum
  JOURNAL   J. Biol. Chem. 272 (15), 9933-9941 (1997)
   PUBMED   9092532
REFERENCE   2  (bases 1 to 628)
  AUTHORS   Mei,B., Komuniecki,P. and Komuniecki,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-MAR-1996) Baisong Mei, Biology, The University of
            Toledo, 2801 W. Bancroft St., Toledo, OH 43606, USA
FEATURES             Location/Qualifiers
     source          1..628
                     /organism="Ascaris suum"
                     /mol_type="mRNA"
                     /db_xref="taxon:6253"
     CDS             12..503
                     /note="high affinity for fatty acids; released by
                     developing larvae into the perivitelline fluid of the egg,
                     and may be involved in maintenance of the impermeable
                     lipid layer of the eggshell and/or sequestration of toxic
                     fatty acids; member of the FABP/P2/CRBP/CRABP family of
                     beta-barrel lipid transporter proteins; does not bind
                     retinol; unusually possesses a cleavable hydrophobic
                     leader"
                     /codon_start=1
                     /product="fatty acid binding protein homolog As-p18"
                     /protein_id="AAA98565.1"
                     /db_xref="GI:1272384"
                     /translation="MRCLVALILTVLIVTPEVEAKTLPDKFLGTFKLERDENFDEYLK
                     ARGYGWIMRQVIKLAGVTKKFRNAASGKPDRYDMENLTTKKDTHHKDWALGEEFQDEA
                     LDSTQHKITFDLKDPNTLTETHIKVDDPTDVETYEYRRDGDYLVMKMSWKGVSTSRYY
                     KKQ"
ORIGIN
        1 ccaagtttga gatgcgctgc cttgtcgctt taattctaac tgtactcatt gtcactcccg
       61 aggttgaagc aaagactcta cccgacaaat tcctcggtac attcaagctt gagcgggatg
      121 aaaacttcga cgaatatctc aaagcaagag gatatgggtg gatcatgaga caggtgatta
      181 agcttgcggg tgttacaaaa aaattccgaa atgcagcatc gggaaagccc gatcgatacg
      241 atatggagaa tctaacgacg aagaaagaca cgcatcacaa ggattgggct ctcggagagg
      301 aattccagga tgaagcactt gacagcactc aacataagat caccttcgat ctgaaagatc
      361 caaacacgct gaccgagacg catattaaag tcgacgatcc aaccgacgta gagacctatg
      421 aatacaggcg tgatggtgac tatctcgtta tgaagatgtc atggaaaggc gtatctacaa
      481 gccgttatta taaaaaacaa taactgaatc catccacgaa taactctcat tttcgaaatt
      541 gcgtaaaaaa ttaccgatat tgacgttgat tatgttatca ttttcgttct gatcatctgt
      601 tattggcgat tgccgcaata aattcgtt
//
```

***Appendix 4 : As-p18 gDNA coding sequence*** [32]

LOCUS AEUI02000026 3850 bp DNA linear INV 09-SEP-2013
DEFINITION Ascaris suum AG00026, whole genome shotgun sequence.
ACCESSION AEUI02000026 REGION: 637232..641081
VERSION AEUI02000026.1 GI:541048163
DBLINK BioProject: PRJNA62057
BioSample: SAMN02953788
Assembly: GCA_000187025.2
KEYWORDS WGS.
SOURCE Ascaris suum (pig roundworm)
ORGANISM Ascaris suum
Eukaryota; Metazoa; Ecdysozoa; Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae; Ascaris.
REFERENCE 1 (bases 1 to 3850)
AUTHORS Wang,J., Czech,B., Crunk,A., Wallace,A., Mitreva,M., Hannon,G.J. and Davis,R.E.
TITLE Deep small RNA sequencing from the nematode Ascaris reveals conservation, functional diversification, and novel developmental profiles
JOURNAL Genome Res. 21 (9), 1462-1477 (2011)
PUBMED 21685128
REFERENCE 2 (bases 1 to 3850)
AUTHORS Wang,J., Mitreva,M., Berriman,M., Thorne,A., Magrini,V., Koutsovoulos,G., Kumar,S., Blaxter,M.L. and Davis,R.E.
TITLE Silencing of germline-expressed genes by DNA elimination in somatic cells
JOURNAL Dev. Cell 23 (5), 1072-1080 (2012)
PUBMED 23123092
REFERENCE 3 (bases 1 to 3850)
AUTHORS Wang,J., Davis,R.E. and Mitreva,M.
TITLE Direct Submission
JOURNAL Submitted (26-JAN-2011) Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, 12801 E. 17th Ave, Aurora, CO 80045, USA
REFERENCE 4 (bases 1 to 3850)
AUTHORS Wang,J., Mitreva,M., Berriman,M., Thorne,A., Magrini,V., Koutsovoulos,G., Kumar,S., Blaxter,M.L. and Davis,R.E.
TITLE Direct Submission
JOURNAL Submitted (21-JUL-2012) Biochemistry and Molecular Genetics, University of Colorado School of Medicine, 12801 East 17th Ave, Aurora, Colorado 80045, USA
COMMENT ##Genome-Assembly-Data-START##
Assembly Method :: Velvet v. 1.1.03
Genome Coverage :: 131.0x
Sequencing Technology :: Illumina GAIIx; Illumina HiSeq
##Genome-Assembly-Data-END##
FEATURES Location/Qualifiers
source 1..3850
/organism="Ascaris suum"
/mol_type="genomic DNA"
/db_xref="taxon:6253"
/sex="male"
/tissue_type="sperm"
gene 1..3850
/locus_tag="ASU_01514"
mRNA join(1..139,391..413,1162..1258,3573..3850)
/locus_tag="ASU_01514"
/product="fatty acid-binding protein-like protein"
ORIGIN
1 atgcgctgcc ttgtcgcttt aattctaact gtactcattg tcactcccga ggtagaagca
61 aagactctac cggacaaatt cctcggtaca ttcaagcttg agcgggatga aaacttcgac
121 gaatatctca aagcaagagg tcaatcttga attgatttgt tgatgagttt ttaattgtaa

```
 181 cgcacgtatg aagcggcctt gcatcgaaag tgtaaaaacg atggtagtga acgaacgatt
 241 ggccgaagcg aaaagaacaa gagatgattg ctctcttttt cggtttccaa aaattcagca
 301 ttctgtgctg atttcgaagt gtgtcatgcc ttctaaaacg agataattgc tgttgtttca
 361 aatatcagaa ttcgcgacca aaagtttcag gatatgggtg gatcatgaga caggtgatta
 421 agcttgcggg tgttacaaaa aaattccgaa atgcagcatc gggaaagccc gatcgatacg
 481 atatggagaa tctaacgacg aagaaagaca cgcatcacaa ggactgggct ctcggagagg
 541 aattccagga tgaagcactt gacagcactc aacataaggc aattatcttc gaccattcta
 601 ggataacaat caaaagcgcc gacatactct gaaataaaag tggtatgacg ctgtgcagat
 661 taaatttagc tgtgaagttc gcagtgactg cagtaatgac actctgatac tcataatcat
 721 ttatagtgta acaggcgtta taccgtcgca attgtagatt cctgagaagc tccttagatc
 781 cggtcgaatt tctgtctgtt tataattctc gaatcaaacg tgtaaaagaa gaaattgatc
 841 gccaacttaa caatcttaca ctgtggcaga tgctatcaat aattgctacg tttgaatgaa
 901 aaacttcaac gaatggctac gtcgcaaaag cacttcggtg acgtgaaatt tttgtgatga
 961 tcgataatat ttatatttag cgtaaatctg atatgcacat aatgttacga cgcggaagac
1021 tagaatgacg catgatgtcc ctcttcagta taaacacggt tgtgcgtttg cgcaaacgag
1081 tcacatcagc gtagtggtca tgaagccaat agctgctttg atctgagaaa tcgaaagaaa
1141 tggaatttga attcaattca gatcaccttc gatctgaaag atccaaacac gctgaccgag
1201 acgcatatta aagtcgacga tccaaccgac gtagagacct atgaatacag gcgtgatggt
1261 gactatctcg ttatggttag ttaacgtttc attcatccta ttttccttgt gcatttttgt
1321 atagtgtcgt aaaaattaag tgagcgttaa aatgttatgc aagtgaaaaa agtgctaggt
1381 gggttaattg ttgttggttt ttaaatttcg tcagatgatc atccgtaatg caccccgtca
1441 gctgtaatgg cagttgagtg tgcaaactta atttcaaatc gtctgaagtt gtttatattt
1501 aaaggcaaca gttgcccgtt tgttcgtcca catcaatcat gaatcactac atgatcctgc
1561 gtgtgtatgt ccaaacgttg acttcattat gagcgtgatc atagagctca catttatctc
1621 tttgcaatgg gcaaacgatt gacttttgca gaagatgtca tggaaaggcg tatctacaag
1681 ccgttactat aaaaaacagt aactgaatcc atccacgaat aactctcatt ttcgaaattg
1741 cctaaaaaat taccgatatt gacgttgatt atgttatcat ttttgttctg atcatctgtt
1801 gttggcgatt gccgcaataa attcattcac aggattttgt tgtcgcacca tcgaacatga
1861 agtcaacaag ttggcgaatg tgtaaaatgg ctagtacatg tcgcttttga aatttttctc
1921 catcaccagc acaaaaatat gggcaatttc tcattcgtca agctcgggag catcattgat
1981 ttttcttacg taagggtcaa atcgtaatgg atgttcggag ttgatgtctg attcaacgtc
2041 tggcttattg atgacatacg ttcgtttgga agcatgagtt cgttctagaa tcatttgagg
2101 agtaatcact ctctatggca ggaatgaaca tgggctaatg tcaggtaata tggggcagaa
2161 atggatgtgg atatgttcgg gggaaggagc aagagagaaa aattctcgta aaacgtaaaa
2221 aagaagagaa gaaataacgt aaaattgcga aagaggtaaa ccacaatctc aacctttgct
2281 aatcgggaga aacaggttat ttaacaaaat ctggtaaatc tattcgctgt attctttcga
2341 taatcctcat aacatccctt aagacctccc ccctcccccc gccccctgcc tcgcgtaaca
2401 ttgacaagga aacaaaattt gcatttaggt aagtacaggt atatgagcgc agtaacaagt
2461 tatgtttgaa aaagcaatga attaattaac gaacgcttta aatgaatcag atcaacaatg
2521 ttcagctaat ggaaaggtta gcgttttcgg acgatgccag ttgataaaaa cggatagatt
2581 ttagtggctt tttcggaaag agtatatctc ggaaggagta tagcttttaa agcgttgctg
2641 taaggaacac attttgtgga ttgcaatgtt tgcactttgt aaatgccctt tcgagccttt
2701 gaatgtgaca ggtgcagatg agagagcaca ataaataacg ctgagaattt tatacgcata
2761 ctcaaatcgg ggttggtcac gcgaactgca aaagttcaag accaaagagt tccgatcgaa
2821 tctgatcatc tttaatgcag ggacaaaaag gagctatgat atactacacg agtagatgct
2881 taaccgcttt cacatgggtg gtcaatacta tctgttcatg atcagtaata ggctgtactc
2941 gctttgcgat gatactgagt aagcttggct atgaagtcca atacggtgga ttacgtcatc
3001 agtgccagta tctgctatct gcctacgagc gagctcgtca cgaagtgctg tcgaatacga
3061 tttgcttata caaatgtacg aatgtacttg tacttgggaa actctcaaat atcgacaaac
3121 gtgcaatact tatgtgatgg caggatcgtt ggcaaagatt atcttgatag gacgatatct
3181 cttgctttta tattcgccca tcatgaacac ctgcgttgac agtgcactga cacttagtac
3241 gttcaaaatt caagatgcgt ctggcatgtc gtaatcgcgg tgaagcgctc ttgagcagga
3301 agatctacag tttcatcaac tcctatgaat acaagcgaat gtttccgttc cttctcctcg
3361 attaaatctt gtggtcgctc gatagagaca aggccgaagt atggtagcat ggcatgatgg
3421 cgctgataac atctgcttgc ggatttgtgt aacactctgt agcagggtac tcagaacagt
3481 ctccacgagc aggttactgc tttaagtgtc gggcaacaat tccgacatca gcttacgaat
3541 cagcgtagca aactgagtct ttaactctcc aggtataacg ggccttaccg ttgtggtgaa
3601 cgaccacgct gcgacacccc tatcgcgcga tgttgtggaa aaaccgcgca gcggctctgc
3661 ttgtacgcat cgaggtcagt gacatctgtt tttctccatt tgacattgta tgcattgcac
3721 tgggttattt gacgatttgg ttgatcccga gatgttcaga ataaacgcca aatgacagtc
3781 tcacatcttc catatacggc aacaacatta aggctggtgg gttgttgtgg aagtctcaaa
3841 ggcattagaa
//
```

***Appendix 5 : Ascaris suum CDP6 gDNA coding sequence*** [32]

```
LOCUS       AEUI02000013          9938 bp    DNA     linear   INV 09-SEP-2013
DEFINITION  Ascaris suum AG00013, whole genome shotgun sequence.
ACCESSION   AEUI02000013 REGION: 634639..644576
VERSION     AEUI02000013.1  GI:541048598
DBLINK      BioProject: PRJNA62057
            BioSample: SAMN02953788
            Assembly: GCA_000187025.2
KEYWORDS    WGS.
SOURCE      Ascaris suum (pig roundworm)
  ORGANISM  Ascaris suum
            Eukaryota; Metazoa; Ecdysozoa; Nematoda; Chromadorea; Ascaridida;
            Ascaridoidea; Ascarididae; Ascaris.
REFERENCE   1  (bases 1 to 9938)
  AUTHORS   Wang,J., Czech,B., Crunk,A., Wallace,A., Mitreva,M., Hannon,G.J.
            and Davis,R.E.
  TITLE     Deep small RNA sequencing from the nematode Ascaris reveals
            conservation, functional diversification, and novel developmental
            profiles
  JOURNAL   Genome Res. 21 (9), 1462-1477 (2011)
   PUBMED   21685128
REFERENCE   2  (bases 1 to 9938)
  AUTHORS   Wang,J., Mitreva,M., Berriman,M., Thorne,A., Magrini,V.,
            Koutsovoulos,G., Kumar,S., Blaxter,M.L. and Davis,R.E.
  TITLE     Silencing of germline-expressed genes by DNA elimination in somatic
            cells
  JOURNAL   Dev. Cell 23 (5), 1072-1080 (2012)
   PUBMED   23123092
REFERENCE   3  (bases 1 to 9938)
  AUTHORS   Wang,J., Davis,R.E. and Mitreva,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JAN-2011) Department of Biochemistry and Molecular
            Genetics, University of Colorado School of Medicine, 12801 E. 17th
            Ave, Aurora, CO 80045, USA
REFERENCE   4  (bases 1 to 9938)
  AUTHORS   Wang,J., Mitreva,M., Berriman,M., Thorne,A., Magrini,V.,
            Koutsovoulos,G., Kumar,S., Blaxter,M.L. and Davis,R.E.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2012) Biochemistry and Molecular Genetics,
            University of Colorado School of Medicine, 12801 East 17th Ave,
            Aurora, Colorado 80045, USA
COMMENT     ##Genome-Assembly-Data-START##
            Assembly Method       :: Velvet v. 1.1.03
            Genome Coverage       :: 131.0x
            Sequencing Technology :: Illumina GAIIx; Illumina HiSeq
            ##Genome-Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..9938
                     /organism="Ascaris suum"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:6253"
                     /sex="male"
                     /tissue_type="sperm"
     gene            complement(1..9938)
                     /locus_tag="ASU_00827"
     mRNA            complement(join(1..1235,2204..2296,2923..3084,4114..4302,
                     4820..4960,6890..7021,7611..7766,8345..8466,9787..9938))
                     /locus_tag="ASU_00827"
                     /product="cell death protein 6"
     CDS             complement(join(1065..1235,2204..2296,2923..3084,
                     4114..4302,4820..4960,6890..7021,7611..7766,8345..8466,
                     9787..9934))
```

/locus_tag="ASU_00827"
/codon_start=1
/product="cell death protein 6"
/protein_id="ERG87327.1"
/db_xref="GI:541048624"
/translation="MTSKGKEIYNTIKRSLGSSSFGVASSSSGSGGVRYSGGTTASKQ
WIHPPDILLNGRVEYSVKMLGVTEVNEPKGTHVIREAIHAIRFQLQVSRSVTGHSGAK
LKKVDLQINVDGVTVVENKTKMVLHKYPLHRISFCADDKQDKRVFSFIAKAENSKRHD
CFVFLSEKLAEQITLTVGEAFDLAYQKFLENNGRELENKKQMIVLRKRIAELETENNE
LKQKLAAALMKDQTNGDAPPPLLPTSPVPNAPPPGLALSPVADVAPGIVPPPPNSRRH
KVLTASTAAIMAELSDSMPKVGRKLENLQIDQMESLFDDEFDPRADERKKAEERRVKD
EFGLDPFGDTFMNDDVSNENSGDTKTGNVEKGHHEPTPEQFERMLSMVDKRLAEMRDG
FATGCLAVGDTGDSARDFDNIYSLPLQSPENENIPSTSRVSNGRS"
ORIGIN
1 gggaaacggg aagtaaataa agacatagcg atgtcttctg tagaaaattt aaccactaag
61 atacgaaaaa aaaaagaaac aaagcttaat accacacaaa atattttaaa atcacaacta
121 tgaagtaaac ctccgaacga cagcagatgt cttggaatat atcacccctc aaataccact
181 ctttttagac gttggaatca caaacatatc gatagcatat cgtttgcctt caaatgaaag
241 ccccccccct caaaaacaaa agacaatcag agtcctcggt gataattatc attatcagct
301 ctcctaaata agctcttctc accaatattc cacttcaata tgcatccacg aatatggcgc
361 attgatgact gcactgttca taccgaagaa acatttccgg gcgtttgagc gctcaccgtt
421 acataaacaa atgcgcgcat aattaatttc acaacaaggt ctcgtgcatt ttttctcgaa
481 attcatttca tcgctaaatt tagttcattt ctctctgctt caacaattca cataagtgtt
541 ttaatatagc ccacaagcac tctcatctag aatgtgagtc gttgatgctg gattccgcat
601 ttcagttcga tgatatatga caacagacag aggcagagga aataattgtg ctgtctatcc
661 cgcttatcac actcttttgc cacagttcca cagaatatct cagttttgtg acgttccctc
721 cacaactgtg tctcctcgtt gtttcgattt gcttatatat ttcgttttct ccatgcaaaa
781 ccacgcatga ctacgaccga ttccgacgaa atgtgtgcat gcaagtttaa aagaggaggg
841 aggaggaggg ggtgggacag agagataaac gaaaaatatc gagaaaagtg aggtcctacc
901 gactgcccgt cctgacatgc ccaaaaccat ccgctcatca ctatccaact gaaatgccca
961 ttgtcacttg tcactgccgc ttcaaagaaa caaatccaaa tcatcggtct gctcttgaaa
1021 tacccaccga aaagtctaaa cagaagtaaa attgatgtgc cttattatga gcgtccattg
1081 gaaacgcgtg atgttgatgg tatgttctcg ttttccggac tctgtagtgg tagactgtaa
1141 atattgtcga aatctcgtgc cgaatcgccc gtatcgccaa cagcgaggca tccggttgcg
1201 aagccatcgc gcatttctgc tagccttttg tctaccttaa acactcttat tagcatattc
1261 aattcaataa gccatcagat acaaatcaaa aaaatcaaac agagaggcca aaattcgtaa
1321 acaggcagag atctcggagt gcattctggt aatgttcaag acgatgcgta atgcgaacac
1381 ggccaacgct gctcgtccga gatgactaag aaagactctg cagaagccat tcggatggca
1441 cactaaacaa agaaaccatg ttttaaataa aatgcagtct tttgagcgcc aattgcagct
1501 gtgatgtgaa acctttgaat agccgtgggc aatttttgga gatgaagcaa ccttttgatc
1561 ggcaggtcgg aaaataccaa gtccagaggt atgtcgcgcg gaaactaatt ttgacaacag
1621 aaacgtgtcc ctgcattaga acgaatggaa tcatatgaat tcttataaga ctgatgaaag
1681 caacaaatct gcggagattt ctgggaggat cgattaagaa agagatatta ctgaagtggc
1741 cgctaatcga gtgcaaaaat agtaaacgac ttttagagat ctgaaacatg caaattttcc
1801 caagtttttt gtaaaaaagt tcgacgaaca taagcatttc caaaaaagag gtgggggggg
1861 ggtggattat tttgtctgtc aatgaattag acaacgtaag acttattcga gagtttaggc
1921 aaaattacaa gaagcaggta tcgttgtgct gccgatcgtt ggtaccggtt taaaatggag
1981 tacctatcga ttacagcgca gctgcagatt ccaatttccc ttcccaggtg aagctgatat
2041 tgctggggaa gaacagttca gtgctgtcat gtcctgcgag atattccgca ggatgtgaca
2101 gaccatcgac aataataaca cctgtttagt gctaatttgt tgagtaatta aaggcgatgg
2161 gtagtggtta cttttcgcag gtaataagca tcctgtcact gaccattgag agcattctct
2221 cgaactgttc aggtgtcggt tcatggtgcc ctttctccac attgcctgtc tttgtatcac
2281 cagagttttc attgctctga aagctcatta ctcagctcct ctacaatgga aactgatttt
2341 cttgaaagca attttttttta attcctcaat ctaaatcgct tctaaagaat aaaaagaaga
2401 aagaaaatgc tccaaagagg ctagtgtgac attattctcc ctctcagccc ttctcatcta
2461 taggctgccc taataatcga aaacagggat gggatgtgct cgataacgca tatcttaaga
2521 aaccgaccgg tcgcacgaac cttcaaggaa aaatgcgctt tcacacaaca tccgacgatc
2581 acctgtgaaa aaaattcggc tgcaaattct tagctggcac ttttaagtgt cagtaagcga
2641 tacagaaaac cactgatgga attaatcgaa ataagaagcg caccacacga cgtaaattat
2701 accaacaaaa tcccattgcc gcaaattcat tttctcgaga actgaggcta tcagacaagc
2761 aggccgacac agatatataa acgcgcagat aaaacatccc cataatgatt aacaaagaac
2821 tgcaaaggcg cataaaatga aggcgctgga agtatgaatg agaatcttat tgaagcagtt
2881 gtatactgtt tagatgtcac taacggagtg atattcaatt accacatcgt cgttcatgaa

2941 ggtgtctccg aacgggtcga gaccaaattc gtccttgact cgtcgctctt cggccttttt
3001 tctctcgtcg gcacgcgggt caaactcatc gtcgaacaaa ctttccatct gatctatctg
3061 aaggttctcg agctttcgtc caacctaaac cacacatttt attacaacca atctcgctca
3121 cttcataaag tatattcttt tataaaagtc aatcgaagaa aagaaaaaat caacgtgaaa
3181 atgcaataaa gatgagaatt cagtgaaaaa gaatgggaaa gttcgttggc atgctatacg
3241 ggcataattt tcatagcacc ccacctgcac tcatcacttt acatgcttta acaccacatc
3301 accagcagcc aaatttggca acctccgcac acggatgtaa gaagccagca gcaagtagca
3361 gatcggcgtt tttggccaaa aacacgatgg gaagtcggcg ctcgaatttt ctgggttcct
3421 ttcgccatag aagtcaatgc aattctgtgc tacccaacac acgttccttt tcttgtattt
3481 gactgataac agccataaac taatgttagc taataatatc ttctcctgca caacttctta
3541 ccttcacttt tacttcaatg cacctccaca tctttattaa taagaacgat atccctagca
3601 ccattaaagg agatgctttt cccacctgtg tgccgctgtt ttgcatactt gccgcttaaa
3661 acaacatcct ttcccgtacg cacacatcct ttaattagcg ttcccctatt agctgtgttg
3721 ataaagcctc actgcatcag aatcgagagc aaggtaaagc acctttttcat atctagatcg
3781 ctgaagtgcc ctacgtcaag gcaacaacct tttccgccac gaaacataac ttcgacagca
3841 atattaagtg cttgagtaat taattccctt ttccttcggc aggttttggt gaggtgaaat
3901 ttgaagatag ctcgcgcctc tccatgcagt ctaatggtaa aaaaagtttt ccgaaaaact
3961 gcggctgcca ataacggctg aagttaacat ctgatatagg tgcacattgt aaataaataa
4021 atttcaatag ttcagcttct cttgatgcaa acgcactagc aagctcagac cacgtcttta
4081 tgaacaatac aaaagcgcat agaaataacg aacttttggc atgctatcgc taagctcagc
4141 catgatggcg gccgttgaag cagtgagtac cttgtgtcga cgtgagttgg gtggtggtgg
4201 tacgatcccg ggtgccacat ccgccactgg ggataacgcc aagcctggag gcggagcatt
4261 tggcactggg ctcgtcggca acaacggtgg tggcgcatca ccctagactt ggcgtttgtc
4321 atcaacttca ggcacagcaa ccatgatata aagcccatat ccacccttcc aattcccttt
4381 ctcccctccc cttcaacaca cacatacgac tgagaaataa ttgaaacagc ttagcaatat
4441 aatttttcct cacttttgga tacctggaaa ctcttttcat ccataattca ctaccgtagt
4501 gtcagaagac tgttttgtga agacgcgtac gaaccattcc gcagtatata atcctggaac
4561 gcaaggccta gataaaccag tctatgaaac tggccaagtg aaaataagat ctgcataaca
4621 gttgatgagg tgaaaaatgc atatttggct gcacaaagcg tcaccaataa tgataaaaat
4681 agcacctaat caacgaagat tttatctaaa atgaaagcac ctatttcgtt aatgatgaaa
4741 tgaaaaaaat aaagcaacag gtaggatgat aagattaggg tgatacccgt agataaatga
4801 taatcagcaa atagctcaca tttgtttgat ccttcattaa tgctgccgca agtttctgtt
4861 ttagctcatt gttctctgtt tcgagttcag cgattcgttt acgtaatacg atcatctgtt
4921 tcttgttctc aagttcacga ccattgttct cgagaaattt ctgcaaaatg atgacctttt
4981 aaactactcg ttatcccagt agactttcat gaagttttta aattcactta aattcaagtt
5041 gcacaacgca tgagacaatg tgatgagaaa aaatgaactt attctcatgc caagacttat
5101 gtaaaatttg agagccctct ggcgtctaat tgctaacata aagaatcatc tccgctatct
5161 atgacgcttc acagttgctg acatacacct cccgctatta tctgatatcg aaagcggatg
5221 tttacctatt agaagaagac aatccgcttg ccgtcgcatt aaaaacgctc ccaaagcgac
5281 gtcagtcagc tagcgatcct cagatgccat ctatcagcaa ataaatccaa aatatgttca
5341 ctcactcaaa catttactca gaattaaccc tcattgcggg caagactgtt tacgcattgt
5401 acccccaaaa caccatactg ctgaatcatt ttattaagct tgaaactctt tgaaacggct
5461 acatacgagc ccttttattc aaatagaggg agatatgtct aacggactca aactttaaga
5521 tgcagatcta tccaccactg tgaattatat tggtgctaat aaatagacgg caattctgtt
5581 gatggtcatc atttggatgg tatctggata gagtggcaag caaccgagct tagaaaaatc
5641 ggcaagtctt cggtggtgag aacccccatg gctgttggcg gtcactgatc aagattgcat
5701 aggtgtcacc gccgataacg ctggaaaaat cggtggccat caatcaccac tcaattttac
5761 tcgataaaaa ccattgctat gtttttaaac agagagctga agatgttata aaacgcatgc
5821 caaaatatgc agaataaaaa tatgccaaaa tatgctgaac atgcaaaata tcattccgca
5881 atgagccata gaatttagcc attgttttta tacgcatcca gaaagaagag cgtaagagga
5941 ggagagggga aggaaaagat ggagaagagc agcgactggg ggagaaagga tggaatggaa
6001 tacagcttca tcaaatagca tatgcttgga tgcgtgaggt caagttcaat ctcattcagc
6061 tatccagaat ttttctctgc gatctcccac agcagttcca atattgcgat tcctattttc
6121 ttatgagacc cttgcatata tttgcaaaag taccccggat cgcactaaaa ctcaattgcc
6181 tgtagattca actggggagc cgggaaagac gtcgatggca tctgacgcaa ataatatctg
6241 ggaaaccaga tcatcttctc gcttatcaat gtttcagcat aataacgaca agaaagaact
6301 ctttcaaatt aatctcgata agaatatgta tttccataaa gtgaacgtgg taccaagagc
6361 tgagtacaac cacatgttat ataacactga tcgttcagcc accgtttagc gtttgtatta
6421 ccgacgactg ccagtatctg atgcactact tttatcgttt tcgtgtgctt atcatatatg
6481 accaccctac gcacttgtgc atgtacgttg agccttcaac accctatata ttgtgcgatt
6541 acaactccct cttcaaagta tttcagcact tcaagagacg tactgtcatg tccggagcag
6601 tcagctgata cattccgttg aataacggat acgcctacat gcctcggtta aggacatctg
6661 cattacgcac acacagtaga tgcaaccatg acttcgatga gacccaccaa ctcatccaaa

6721 acacaacgct tgatctcggc cgggattgac cagacgaccc ctgcgtgtga agcaggcgtg
6781 ataaccgcta caccacgaga cctcccctct ctcttcctgt gctgcatcat cgactagtac
6841 aagccataac tgctttatcc aatgacccaa gcaattgtga ataaatcacc tgatatgcaa
6901 ggtcgaacgc ttcgccaacg gtgagcgtta tctgctcggc gagcttttcg ctcagaaata
6961 caaagcaatc gtgccgcttt gagttttcag ccttcgcgat gaatgaaaac actcgtttgt
7021 cctgaaattt tgagcaaaca tcactacaca tcttaacaaa caaattaagt atctgggcgc
7081 attttccttt taaagcctgt ttaaagctag atatctgcat tttggtggtg aatgaagcag
7141 cggtggggtt tggaaagggg aaggtgaagg ggatggagag gtgagagtag atatgtggaa
7201 aggagagagc agaagatgtg ggaaggagag aatgtggaga attcaacatc gctaaaggac
7261 atccaccaat tagggaaatt ccacaggtga agtcgaactg acaaactctt tttttgagcc
7321 aaactgaatt aacgacaagc gaattcaatc ttttaaagtg acatcaacga caacgccaaa
7381 agagaatatt ttgaagcaag catgtcgctg ctaatcgaat gtaaagctga atgacttaag
7441 gaaggaaaca atcggcatcg gatatgagct ctcataaata ttctatttta gctattctca
7501 taatgcaatt tcaactgaaa aaagctccga tgactgatta ctcttgatcg aaaagaggaa
7561 ataaaatgga tgacaactga acagaaaaaa ttcgaaagaa attgttgaac ctgcttatcg
7621 tcggcgcaga acgaaatcct atgtagagga tacttatgca aaaccatttt tgttttgttt
7681 tccaccactg tcacaccgtc cacgttaatt tgtaagtcaa ctttcttcaa tttggcgccg
7741 gaatgccccg ttacgctgcg actgacctga aatttgagta attgatgact ggttgacatg
7801 acatctcata tacatggttt atcggtcata gccatcaacg gcttaaatag gtgaataaaa
7861 ttacacactg ctctatcttc acagtaacga gtgcgtctca attaaagtcg aaaacaacgc
7921 agatggtgtg aaattttcta agacgaaagt aggtcggtgt cgttcacaac cggaatgcga
7981 ttcccgtaag atttgtggtt ctgttcttcg atattaaacc tcatataaga gaggaatgac
8041 aggaaatggt cggatagaaa taaatttcct actggttcga atgcaggtga aactcgcatt
8101 ttctaacaca aaactttcgc agggagtaaa aaaactaata tatgtagagg agcaaaataa
8161 agaacattct ttgcacaaca ccgaactgct ttaaaattca atcgacgaga ggattatagg
8221 agatattcta aggacaaaac aaagttctca gtccacacca cacagtaacc gaaagtcaga
8281 tttcgtagta gcgaactaga gctcaacggc atttaacgag caatgtcaag taccggaaac
8341 caacctgcag ttggaatctg atggcgtgaa tggcctcacg tatcacgtgt gttcccttcg
8401 gttcattcac ctctgttacg ccaagcatct tcaccgaata ctcaacgcga ccatttagaa
8461 gtatatctgc aaaagcaaac tagctaccct caaggaaacg tcctgctttg tgcacgacac
8521 agctactgca tgagtcttta tgcagcactg aagcaaagag cttagagtgt tgggctcgag
8581 ctccagcacg accaaaccta ccgatatggc gtctcaccac tccataaaat actacatctt
8641 ctgcatcgta cgcatttctg ctgctcaggt tgcggaagca ataaattttc atcctttagc
8701 tagcactgtg acgaggaagg tccttccttc ttttacaggg aaatttcccc attcccaatt
8761 aaaaatcccc ttcgtttgca aattctgcta ttcccctccct ttatttgtcc gtttgcccta
8821 tcgggaatag aagaaagcaa tttgctatga aattttcact ccttttgtag aaaacaaatc
8881 gtactgttta acacttcgcg aaatggaaac cactgtgcga atgataaaac atttcggtga
8941 ggctattcat cccttacaaa tcgccttttc tctatgctat ccacattcga atttctattt
9001 catttctctg ttttgaacaa aaagaaccta aagggaattc tccacgtcca ctcctcctcc
9061 tttctatttc actctcagat gtaatatatg ttcgcatata atcacgatcg aaactgaaat
9121 gatttagcaa attgctgcaa tccactagaa tcttttcagt cttgatcaca tcgattgctt
9181 gaatttgagg agaaaaaaag gagaaaaagg gtggagggag ggagagggaa cagcagaatg
9241 agtcagaaat ggcattatga tgattttatt caaatcgaat gcattcactt cagtcatatt
9301 tcaccaccta ttagtttata agagtcgtat ccactatatt acctttcttc acaagcgaac
9361 actcataact atagtctgaa agtggaatgc aatttgcata tttaaatgcc atctaaacga
9421 ctgctcccat tccgttaccg agacagaatg cacacaatat cacatattgc gacgatacaa
9481 tcgtttcagc gatcgtccca atgcacctta ctgactaaga ttataccaaa tattggtgaa
9541 atcgtggaca taccagagtt gataatttca ataccttcta ttatgtggcc ttcattattc
9601 atgcaacatt acctacatca ctttcacaaa ctatttcacg actgacaggg cggaaatctt
9661 gttgaagggc aactgttagt cgacaataaa cctgcacgtc cgaccgaaag atcgcaaagt
9721 accttatcca gctccactaa cgaaaaaaca ccacagcaaa tgggcaggta acaactggaa
9781 atgaacctgg tggatggatc cattgtttgc tagctgttgt accgcctgaa taacgcacac
9841 cgccagatcc gctcgaagaa cttgccactc cgaacgaaga cgatcccaac gatcgcttta
9901 ttgtattata tatttctttg cctttggatg tcatctgc
//

## ***Appendix 6: RNA extraction protocol of LBE***

***TRIzol-based RNA extraction protocol with Zymo columns***

(References: Prat *et al*., FEBS J., 2012; Buttet et al., unpublished)

Material

- TRIzol reagent (Invitrogen, 15596-026, 100 ml, CHF 409.-)
- Chloroform.
- DEPC-treated water.
- 100% ethanol.
- RNA purification kit: Direct-zol RNA MiniPrep (Zymo Research)
- RQ1 DNase kit (Promega)
- Table-top Eppendorf refrigerated centrifuge.
- BioSphere RNase-free 1.5 ml tubes.

Procedure

*A. Sample lysis and extraction*

1. Thaw cell suspension (in LifeGuard™) at RT.
2. Centrifuge the sample at 10000 rpm for 5 min at 4°C. Remove the supernatant.
3. Add 0.5 ml of TRIzol and resuspend the pellet by repetitive pipetting.
4. Incubate sample for 5 min at RT for complete lysis.
5. Add 0.1 ml of chloroform.
6. Vortex the sample vigorously for 15 s.
7. Incubate for 2 min at RT.
8. Centrifuge the sample at 12000 x g (10600 rpm) for 15 min at 4°C (cold centrifuge).
   **Note**: the sample separates into an upper colourless aqueous phase containing the RNA and a lower phenol-containing red phase (containing DNA and proteins).
9. Recover 400 ml of the upper phase by careful pipetting and transfer to a new tube. Avoid taking any of the interphase or organic phase.

*B. RNA preparation*

1. To the aqueous phase obtained in *A9*, add 0.4 ml of 100% ethanol. Vortex briefly.
2. Load the mixture on a Zymo column.
3. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
4. Transfer the column in a fresh collection tube.
5. Add 400 ml of RNA Prewash buffer.
6. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
7. Discard the flow-through.
8. Add 700 ml of RNA Wash buffer.
9. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
10. Discard the flow-through.
11. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
12. Transfer the column in a RNA-free Eppendorf tube.
13. Add 50 ml of Elution buffer.
14. Incubate 1 min.
15. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
16. Keep the eluted RNA on ice.
17. Remove 5 µl for DNA test

*C. DNase treatment*

1. To 45 ml sample, add 25 ml of DEPC water, 10 ml of RQ1 DNase 10x buffer and 20 ml of RQ1 DNase, respectively.
2. Incubate at 37°C for 2 h.
3. Add 10 ml of RQ1 DNase Stop solution. Vortex briefly.
4. Incubate at 65°C for 10 min.

*D. RNA recovery*

1. Add 90 ml of DEPC water to the DNase-treated sample
2. Add 200 ml of 100% ethanol.
3. Vortex briefly
4. Load the mixture on a Zymo column.
5. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
6. Transfer the column in a fresh collection tube.
7. Add 400 ml of RNA Prewash buffer.
8. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
9. Discard the flow-through.
10. Add 700 ml of RNA Wash buffer.
11. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
12. Discard the flow-through.
13. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
14. Transfer the column in a RNA-free Eppendorf tube.
15. Add 25 ml of Elution buffer.
16. Incubate 1 min.
17. Centrifuge at 12000 x g (10600 rpm) for 1 min at 4°C (cold centrifuge).
18. Keep the eluted RNA on ice.
19. Proceed with RNA quantification and reverse transcription readily.

***Appendix 7: Reliaprep™ RNA Tissue Miniprep System quick protocol***

# ReliaPrep™ RNA Tissue Miniprep System

INSTRUCTIONS FOR USE OF PRODUCTS Z6110, Z6111 AND Z6112.

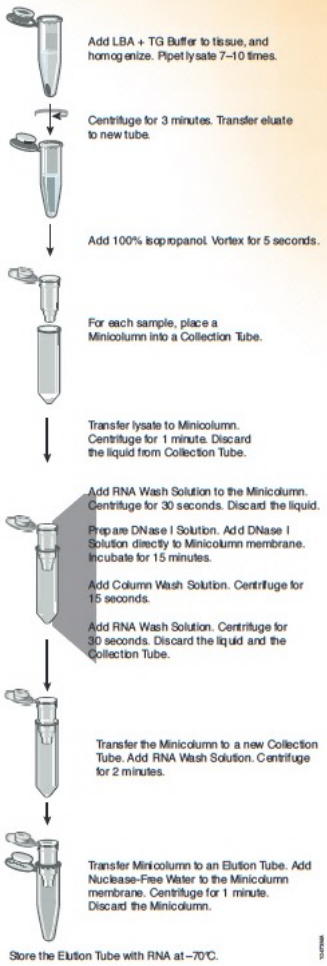
Quick PROTOCOL

## Protocol for Isolation of RNA from Non-Fibrous Tissue

1. Before beginning the ReliaPrep™ RNA Tissue Miniprep protocol, see **Section 4.A., Preparation of Solutions**, in the *ReliaPrep™ RNA Tissue Miniprep System Technical Manual*, #TM394. For best results, Prepare the four required solutions immediately prior to use.
2. Verify that 1-Thioglycerol has been added to the LBA Buffer. Add LBA + TG Buffer to the tissue sample in accordance with the table below.

| Tissue Input Range | LBA + TG Buffer | 100% Isopropanol |
|---|---|---|
| ≤5mg | 250µl | 85µl |
| >5mg | 500µl | 170µl |

3. Disrupt up to 20mg of sample using a tissue homogenizer, followed by pipetting 7–10 times to shear the DNA using a P200 or P1000 pipettor.
4. Clear homogenates by centrifugation for 3 minutes at 14,000 × *g*, then transfer them to a clean tube.
5. Add Isopropanol as recommended in the table above. Mix by vortexing 5 seconds.
6. Wearing gloves, unpack one Minicolumn, two Collection Tubes and one Elution Tube for each sample. Label each tube and Minicolumn. Place one Minicolumn into a Collection Tube for each sample.
7. Transfer lysate to a Minicolumn in a Collection Tube. Centrifuge at 12,000–14,000 × *g* for 1 minute at 20–25°C.
8. Remove the ReliaPrep™ Minicolumn, and discard liquid in the Collection Tube. Replace the Minicolumn in the Collection Tube. Add **500µl of RNA Wash Solution** to the Minicolumn. Centrifuge at 12,000–14,000 × *g* for 30 seconds. Empty the Collection Tube, and place it in the microcentrifuge rack.




ORDERING/ TECHNICAL INFORMATION:
www.promega.com □ Phone 608-274-4330 or 800-356-9526 □ Fax 608-277-2601



Promega

Printed in USA Revised 4/14.
Part #9FB145

## Protocol for the Isolation of RNA from Non-Fibrous Tissue (continued)

9. Prepare **DNase I Incubation mix** by combining the following amounts of reagent, per sample, *in the order listed:*

| Solution | Volume × | Number of Preps | = Total |
|---|---|---|---|
| Yellow Core Buffer | 24µl | | |
| $MnCl_2$, 0.09M | 3µl | | |
| DNase I | 3µl | | |

   Mix by gently pipetting; **do not vortex**. The volumes listed above make enough DNase I mix for a single sample. Multiply this amount by the number of samples to calculate the amount of DNase I mix to prepare.

10. Apply **30µl of DNase I Incubation mix** to the Minicolumn membrane. Incubate for 15 minutes at 20°–25°C.
11. Add **200µl of Column Wash Solution** (with ethanol added) to the Minicolumn. Centrifuge at 12,000–14,000 × *g* for 15 seconds.
12. Add **500µl of RNA Wash Solution** (with ethanol added). Centrifuge at 12,000–14,000 × *g* for 30 seconds. Discard the wash solutions and the Collection Tube.
13. Place the ReliaPrep™ Minicolumn into a new Collection Tube. Add **300µl** of **RNA Wash Solution** and centrifuge at high speed for 2 minutes.
14. Transfer the ReliaPrep™ Minicolumn from the Collection Tube to an Elution Tube. Add **Nuclease-Free Water** to the Minicolumn membrane as recommended in the table below. Place the Minicolumn and Elution Tube into a centrifuge with the Elution Tube lid facing to the outside. Centrifuge at 12,000–14,000 × *g* for 1 minute.

| Tissue Input Range | Nuclease-Free Water |
|---|---|
| 5mg or less | 15µl |
| 5 to 20mg | 30µl |

15. Discard the Minicolumn. Cap the Elution Tube containing the purified RNA and store at –70°C.

Detailed protocol information is available in the *ReliaPrep™ RNA Tissue Miniprep System Technical Manual* #TM394, available at: **www.promega.com/protocols/**

ORDERING/ TECHNICAL INFORMATION:
www.promega.com □ Phone 608-274-4330 or 800-356-9526 □ Fax 608-277-2601



Printed in USA. Revised 4/14.
Part #9FB145

***Appendix 8: Reliaprep™ RNA Cell Miniprep System quick protocol***

# ReliaPrep™ RNA Cell Miniprep System

INSTRUCTIONS FOR USE OF PRODUCTS Z6010, Z6011 AND Z6012.

Quick PROTOCOL

## Protocol

1. Before beginning the ReliaPrep™ RNA Cell Miniprep protocol, see **Section 4.A., Preparation of Solutions** in the *ReliaPrep™ RNA Cell Miniprep System Technical Manual*, #TM370. Prepare the four required solutions immediately prior to use, for best results.
2. To harvest adherent cells, use the protocol in Section 8.A., TM370, prior to cell lysis. For suspension cells proceed to Step 3, below.
3. Collect cells in a sterile centrifuge tube by centrifugation at 300 × *g* for 5 minutes.
4. Wash the cell pellet with ice-cold, sterile 1X PBS. Centrifuge at 300 × *g* for 5 minutes. Carefully discard the supernatant.
5. Add BL + TG Buffer to the washed cell pellet (see the table, below). If frozen cell pellets are used as starting material, add BL + TG Buffer to the frozen pellets before thawing.

| Number of Cells | BL + TG Buffer | 100% Isopropanol |
|---|---|---|
| $1 \times 10^2$ to $5 \times 10^5$ | 100µl | 35µl |
| $>5 \times 10^5$ to $2 \times 10^6$ | 250µl | 85µl |
| $>2 \times 10^6$ to $5 \times 10^6$ | 500µl | 170µl |

6. Disperse the cell pellet and mix well by vortexing and/or pipetting.
   **Note:** After adding BL + TG Buffer, pipet 7–10 times to shear the DNA. For >2 × $10^6$ cells, pass the lysate through a 20-gauge needle 4–5 times to shear the genomic DNA.
7. Add Isopropanol as recommended in the table above. Mix by vortexing 5 seconds.
8. Wearing gloves, unpack one Minicolumn, two Collection Tubes and one Elution Tube for each sample. Label each tube and Minicolumn. Place one Minicolumn into a Collection Tube for each sample.
9. Transfer lysate from Step 7 to a Minicolumn in a Collection Tube. Centrifuge at 12,000–14,000 × *g* for 30 seconds at 20°–25°C.
10. Remove the ReliaPrep™ Minicolumn and discard liquid in the Collection Tube. Replace the Minicolumn in the Collection Tube. Add **500µl of RNA Wash Solution** to the Minicolumn. Centrifuge at 12,000–14,000 × *g* for 30 seconds. Empty the Collection Tube.

Add BL + TG Buffer to cell pellet and mix well by vortexing or pipetting. (To harvest adherent cells, see Section 8.A.)

Add 100% isopropanol. Vortex for 5 seconds.

For each sample, place a Minicolumn into a Collection Tube.

Transfer lysate to Minicolumn. Centrifuge for 30 seconds. Discard the liquid from Collection Tube.

Add RNA Wash Solution to the Minicolumn. Centrifuge for 30 seconds. Discard the liquid.

Prepare DNase I Solution. Add DNase I Solution directly to Minicolumn membrane. Incubate for 15 minutes.

Add Column Wash Solution. Centrifuge for 15 seconds.

Add RNA Wash Solution. Centrifuge for 30 seconds. Discard the liquid and the Collection Tube.

Transfer the Minicolumn to a new Collection Tube. Add RNA Wash Solution. Centrifuge for 2 minutes.

Transfer Minicolumn to an Elution Tube. Add Nuclease-Free Water to the Minicolumn membrane. Centrifuge for 1 minute. Discard the Minicolumn.

Store the Elution Tube with RNA at –70°C.

10472MA

ORDERING/TECHNICAL INFORMATION:
www.promega.com □ Phone 608-274-4330 or 800-356-9526 □ Fax 608-277-2601



Promega

Printed in USA 3/12.
Part #9FB131

## Protocol (continued)

11. Prepare **DNase I incubation mix** by combining the following amounts of reagent, per sample, *in the order listed*.

| Solution | Volume | × | Number of Preps | = | Total |
|---|---|---|---|---|---|
| Yellow Core Buffer | 24µl | | | | |
| $MnCl_2$, 0.09M | 3µl | | | | |
| DNase I | 3µl | | | | |

Mix by gently pipetting; **do not vortex**. The volumes listed above make enough DNase I mix for a single sample. Multiply this amount by the number of samples to calculate the amount of DNase I mix to prepare.

12. Apply **30µl of DNase I incubation mix** to the Minicolumn membrane. Incubate for 15 minutes at 20°–25°C.
13. Add **200µl of Column Wash Solution** (with ethanol added) to the Minicolumn. Centrifuge at 12,000–14,000 × *g* for 15 seconds.
14. Add **500µl of RNA Wash Solution** (with ethanol added). Centrifuge at 12,000–14,000 × *g* for 30 seconds. Discard the wash solutions and the Collection Tube.
15. Place the ReliaPrep™ Minicolumn into a new Collection Tube. Add **300µl** of **RNA Wash Solution** and centrifuge at high speed for 2 minutes.
16. Transfer the ReliaPrep™ Minicolumn from the Collection Tube to an Elution Tube. Add **Nuclease-Free Water** to the Minicolumn membrane as recommended in the table, below. Place the Minicolumn and Elution Tube into a centrifuge with the Elution Tube lid facing to the outside. Centrifuge at 12,000–14,000 × *g* for 1 minute.

| Number of Cells | Nuclease-Free Water |
|---|---|
| $1 \times 10^2$ to $5 \times 10^5$ | 15µl |
| $>5 \times 10^5$ to $2 \times 10^6$ | 30µl |
| $>2 \times 10^6$ to $5 \times 10^6$ | 50µl |

17. Discard the Minicolumn. Cap the Elution Tube containing the purified RNA and store at –70°C.

Detailed protocol information is available in the *ReliaPrep™ RNA Cell Miniprep System Technical Manual* #TM370, available at:
**www.promega.com/protocols/**



Promega
Printed in USA 3/12..
Part #9FB131

***Appendix 9: BioScript All-in-One cDNA Synthesis SuperMix protocol***

## 1. Notice!

- This product is designed for use in real-time quantitive PCR (RT-qPCR).
- Digestion of genomic DNA before cDNA synthesis is strongly recommended.

## 2. General Information

BioScript All-in-One cDNA Synthesis SuperMix provides first-strand cDNA synthesis, optimized with the high-temperature capability of Reverse Transcriptase and an optimized SuperMix formula. In one tube, the pre-blended 5 × qRT SuperMix contains all the necessary components for reverse transcription of YOUR RNA template. The resulting cDNA product is suitable for use in real-time quantitative RT-PCR (qRT-PCR).

## 3. Kit Components

| Component | B24403 | B24408 |
|---|---|---|
| 5 × qRT SuperMix* | 400 µL | 1 mL × 2 |
| 5 × No RT ControlMix‡ | 40 µL | 200 µL |
| RNase-free Water | 1 mL × 2 | 1mL × 10 |

* contains Buffer, dNTP, Reverse Transcriptase, RNase inhibitor, Random primers: Oligo dT primer mix.
‡contains all the components in 5 × qRT SuperMix, except Reverse Transcriptase.

## 4. Storage and Quality Guarantee

Store at -20°C for up to one year. It is recommended to pre-aliquot the mix into small batches for frequent usage. Product quality is guaranteed under proper storage conditions.

## 5. Protocol for cDNA Synthesis

The following protocol has been optimized for generating first-strand cDNA for use in qRT-PCR.

a) Combine the following components in a tube on ice.

| Component | Volume |
|---|---|
| Total RNA/mRNA | 50 ng-5µg/5-500 ng |
| 5× qRT SuperMix | 2 µL |
| RNase-free Water | Up to 10 µL |

Note: Digestion of genomic DNA with DNase I beforehand is strongly recommended.

No RT control (optional)

| Component | Volume |
|---|---|
| Total RNA/mRNA | 50 ng-5μg/5-500 ng |
| 5× No RT ControlMix | 2 μL |
| RNase-free Water | Up to 10 μL |

Note: The reaction is for detection of genomic DNA contamination recommended in RNA template.

b) Gently mix tube contents and incubate at 42 °C for 15 minutes (for quick procedure).

c) Incubate tube at 85 °C for 2 minutes to inactivate BioScript All-in-One RT/RI. Chill on ice until contents reach room temperature.

d) Store at -20 °C until use.

For standard procedure:

To obtain cDNA of higher quality, incubate the tube at 25 °C for 10 minutes, then at 42 °C for 30 minutes for extension, and finally at 85 °C for 5 minutes to terminate the reaction.

## 6. Important Points While Using This Product (Please Read Carefully)

a) Gently invert the tube upside down several times before use. Do NOT vortex. Brief centrifugation prior to use is recommended.

b) High-quality RNA template is recommended to ensure successful cDNA synthesis.

c) To eliminate false positive results by genomic DNA contamination, digestion of template RNA with DNase is strongly recommended.

d) Mixing all reaction components in one step will result in a successful reverse transcription reaction.

e) For RNA templates of complex structure or to achieve higher synthesis efficiency of standard RNA templates, extend the incubation of template and primer as described.

***Appendix 10: PowerLyzer® UltraClean® Tissue and Cells RNA isolation kit***


## Important Notes Before Starting

### Information

- Solution TR1 may form a precipitate. Warm to 37°C for 5-10 minutes to dissolve.
- Prepare Solution TR1 by adding β- mercaptoethanol (βME)
  - Add 10 µl of β- mercaptoethanol (βME) for every 1 ml of the Solution TR1 for all samples to be processed.
    **Note:** Prepare Solution TR1 in smaller aliquots with fresh βME according to the number of samples you need to process that day instead of adding βME to the whole bottle. Use a fume hood when opening βME to avoid exposure to the chemical.
    **Note:** βME will be stable in lysis buffer up to one month. You may add fresh βME to the lysis buffer if necessary.
- Perform all steps at room temperature (15-30°C). Use a standard microcentrifuge. If using a refrigerated centrifuge, do not allow the centrifuge to cool.
- Homogenized samples in Solution TR1 may be stored at -80°C for up to 2 months until ready to use.

**Table 2. Use the amount of Solution TR1 lysis buffer as specified in the table below:**

| Amount of starting material | Volume of Solution TR1 |
|---|---|
| Tissue ≤ 12 mg | 300 µl |
| Tissue > 12 mg - 25 mg | 600 µl |
| Cells ≤ 5 x $10^6$ | 300 µl |
| Cells 5 x $10^6$- 1 x $10^7$ | 600 µl |

For spleen and thymus, do not use more than 10 mg of tissue per 300 µl of Solution TR1 to help decrease genomic DNA contamination. For liver samples, do not exceed 20 mg of tissue or you may exceed the column binding capacity. Up to 100 µg of RNA may be recovered from a single column. For spleen, thymus and tissue culture cells, genomic DNA removal using the On-Spin Column DNase I Kit (MO BIO Catalog# 15100-50) is strongly recommended.

### Bead Tube Identification

Due to the high energies of the PowerLyzer® 24, the potential of marring of the tops of the caps is possible, therefore it is recommended to mark the sides of the Ceramic Bead Tubes as well as the caps to ensure proper sample identification.

### Homogenization Methods

The PowerLyzer® UltraClean® Tissue & Cells RNA Isolation Kit is optimized using the PowerLyzer® 24 bead beater as the homogenizer. The optimal setting is two cycles of 45 seconds at 3500 RPM with a 30 second dwell in between cycles. Samples can be kept chilled in a bench top cooler or on ice before homogenization to protect the RNA integrity while processing.

## Experienced User Protocol

**Please wear certified RNase-Free gloves (Catalog#1556) at all times.**

**Note:** See Important Notes Before Starting section on the previous page for preparation of Solution TR1.

1. Properly identify each Ceramic Bead Tube on both the cap and on the side; See "**Important Notes Before Starting**" for more information.

2. Homogenize tissue samples following ONLY one of the methods a-d described below. For alternative homogenization methods contact MO BIO Laboratories Technical Services (technical@mobio.com).

   **a) PowerLyzer® 24 or other bead beater**

   In a PowerLyzer® Ceramic Bead Tube, 2.8 mm (provided), add either **300 µl or 600 µl of Solution TR1** containing βME, according to Table 2. Chill the tubes on ice or in a cooling block.
   1) Weigh the tissues and add them to the Bead Tube. Keep chilled until ready to homogenize.
   2) Place Bead Tubes into the Tube Holder of the PowerLyzer® 24. The tubes must be balanced (evenly spaced) on the Tube Holder. Homogenize the tissue for 2 cycles at speed 3500 rpm for 45 seconds each with a 30 second dwell between cycles.
   3) Centrifuge the Bead Tubes containing the tissue lysate at 13,000 x *g* for 1 minute to collect the lysate. Transfer the lysate to a new 2 ml Collection Tube (provided).

   **b) Rotor-stator or Polytron homogenizer**
   1) Weigh the tissues and place into an appropriate sized vessel for your homogenizer.
   2) Add **300 µl or 600 µl of Solution TR1** to the vessel, according to Table 2.
   3) Homogenize for 30-40 seconds until the tissue is completely liquefied and no visible particulates remain.
   4) Transfer the lysate to a new 2 ml Collection Tube (provided).

   **c) Liquid nitrogen and mortar and pestle**
   1) Weigh the tissues and place into the pre-chilled mortar.
   2) Add liquid nitrogen and homogenize the tissue to a fine powder.
   3) Resuspend the powdered tissue with **300 µl or 600 µl of Solution TR1,** according to Table 2, and transfer to a 2 ml Collection Tube (provided).
   4) Shear genomic DNA using a 20 gauge needle on a 1 cc syringe by moving the lysate in and out of the syringe at least 10 times or more until the sample loses viscosity.

   **d) Homogenization of cells**
   1) Collect cells from culture medium and perform a cell count to determine the correct volume of **Solution TR1** to use.
   2) Pellet cells at 2000 x *g* for 5 minutes and wash the cells once with phosphate buffered saline to remove the culture medium. Pellet the cells again at 2000 x *g* for 5 minutes.
   3) Add **300 µl or 600 µl of Solution TR1** to the cell pellet according to Table 2 and transfer the sample to a 2 ml Collection Tube (provided).
   4) Vortex for 2 minutes to resuspend and homogenize the cells. No visible cell debris should remain.

3. Add **1 equal volume (300 µl or 600 µl) of Solution TR2** to the lysate and mix by pipetting.

4. Transfer 600 µl of lysate to the Spin Filter. Centrifuge for 1 minute at ≥10,000 x *g*. Discard the flow-through and place the Spin Filter back into the 2 ml Collection Tube. If you used 600 µl of Solution TR1 and Solution TR2, repeat with a second loading of lysate. Centrifuge for 1 minute at ≥ 10,000 x *g* and discard the flow-through. Place the Spin Filter back into the same 2 ml Collection Tube.
5. Wash the Spin Filter with **500 µl of Solution TR3**. Centrifuge for 1 minute at ≥ 10,000 x *g*. Transfer the Spin Filter to a new 2 ml Collection Tube (provided).

   **Note:** An optional on-column DNA removal procedure may be inserted after this step of the protocol. See page 13 for instructions.

6. Wash the Spin Filter with **500 µl of Solution TR4**. Centrifuge for 1 minute at ≥ 10,000 x *g*. Discard the flow-through and place the Spin Filter back into the same 2 ml Collection Tube.
7. Repeat step 6.
8. Centrifuge the Spin Filter in the empty 2 ml Collection Tube for 2 minutes at 13,000 x *g* to dry the membrane. Transfer the Spin Filter to a new 2 ml Collection Tube (provided).
9. To elute the RNA, add **50-100 µl of Solution TR5** directly onto the Spin Filter membrane and allow it to incubate for 1 minute at room temperature. Centrifuge for 1 minute at ≥ 10,000 x *g*.

   We recommend storing RNA frozen (-20°C or -80°C). RNase-Free Water contains no EDTA.

**Thank you for choosing the PowerLyzer® UltraClean® Tissue & Cells RNA Isolation Kit.**

***Appendix 11: RNA PowerSoil total RNA isolation kit***


## Protocol for LifeGuard™ Soil Preservation Solution

1. Add between 2 and 2.5 volumes of LifeGuard™ Soil Preservation Solution per gram of soil. For example 1 gram of soil would require 2 to 2.5 ml of LifeGuard™. If you are collecting the soil directly into the RNA PowerSoil® 15 ml Bead Tube, add 5 ml of LifeGuard™ into a 2 gram soil sample.
   **Note**: If you are working with sediment samples, use 3 volumes of LifeGuard™ per gram of wet weight.
2. Vortex or hand-mix the solution and soil until the entire sample is saturated with solution. Excess LifeGuard™ Solution should be sitting on top of your soil sample.
3. Store at -20°C, 4°C, or room temperature as desired. When ready to process the RNA, centrifuge the sample at 2500 x *g* for 5 minutes to collect the soil. Remove the LifeGuard™ Soil Preservation Solution from the tube.
4. If you collected soil directly in the 15 ml Bead Tube, you can remove the LifeGuard™ after centrifugation and continue at step 2 of the RNA PowerSoil® Total RNA Isolation Kit protocol.
5. If you stabilized soil in another vessel, weigh the amount of soil you need and transfer to the 15 ml Bead Tube, or a conical tube, and then centrifuge to remove the LifeGuard™ Solution.

A number of different soils have been tested using LifeGuard™ and each soil will differ in its biomass and its saturation with water. Sediment samples require a higher volume of LifeGuard™ (3:1) due to dilution of the reagent.

Biomass content may play a role in the stabilization of soils at different temperatures and the ability of LifeGuard™ to freeze the community profile. For a soil with unknown microbial content or where temperatures may exceed room temperature (22-25°C), it may be preferable to store the soil at -20°C or 4°C to ensure the original community profile is maintained and to use more than 2.5 ml of LifeGuard™ per gram of soil. For long term storage and transport (>30 days), store stabilized soil at -20°C.

## Experienced User Protocol

**Wear RNase-Free Gloves (1556) at all times and remove RNase from the work area using Lab Cleaner (12095) for RNase Removal. Both of these products are available from MO BIO. Please see the "Products recommended for you" section at the end of this manual.**

1. Add up to 2 g of soil to the 15 ml **Bead Tube** (provided).
   **Note:** Please refer to Hints and Troubleshooting Guide for information regarding the amount of soil to process.

2. Add 2.5 ml of **Bead Solution** to the Bead Tube followed by 0.25 ml of **Solution SR1** and 0.8 ml of **Solution SR2**.

3. Add 3.5 ml of **phenol:chloroform:isoamyl alcohol** (pH 6.5 – 8.0, [User supplied]) to the bead tube, cap and vortex to mix until the biphasic layer disappears.

4. Place the Bead Tube on the Vortex Adapter (MO BIO Catalog # 13000-V1-15) and vortex at maximum speed for 15 minutes.

5. Remove the Bead Tube from the Vortex Adapter and centrifuge at 2500 x *g* for 10 minutes at room temperature.

6. Remove the Bead Tube from the centrifuge and carefully transfer the upper aqueous phase (avoiding the interphase and lower phenol layer) to a clean **15 ml Collection Tube** (provided). The thickness of the interphase will vary depending on the type of soil used. Discard the phenol: chloroform:isoamyl alcohol in an approved waste receptacle.
   **Note:** The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.

7. Add 1.5 ml of **Solution SR3** to the aqueous phase and vortex to mix. Incubate at 4°C for 10 minutes.

8. Centrifuge at 2500 x g for 10 minutes at room temperature. Transfer the supernatant, without disturbing the pellet (if there is one), to a new **15 ml Collection Tube** (provided).

9. Add 5 ml of **Solution SR4** to the Collection Tube containing the supernatant, invert or vortex to mix, and incubate at room temperature for 30 minutes.
   **Note:** The previous protocol instructions were to incubate at -20°C for 30 minutes. If you've used the -20°C incubation before and know that your soil type yields good results at that temperature, you may continue to follow that protocol.

10. Centrifuge at 2500 x *g* for 30 minutes at room temperature.

11. Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 minutes.
    **Note:** Depending on soil type, the pellet may be large and/or dark in color.

12. Shake Solution SR5 to mix. Add 1 ml of **Solution SR5** to the 15 ml Collection Tube and resuspend the pellet completely by repeatedly pipetting or vortexing to disperse the pellet.

**Note**: Depending on the soil type, the pellet may be difficult to resuspend. Resuspension may be aided by placing the tubes in a heat block or water bath at 45°C for 10 minutes, followed by vortexing. Repeat until the pellet is resuspended.

13. Prepare one **RNA Capture Column** (provided) for each RNA Isolation Sample:
    a. Remove the cap of a **15 ml Collection Tube** (provided) and place the **RNA Capture Column** inside the **15 ml Collection Tube**. The column will hang in the **15 ml Collection Tube**.

    b. Add 2 ml of **Solution SR5** to the **RNA Capture Column** and allow it to gravity flow through the column and collect in the 15 ml Collection Tube. Allow **Solution SR5** to completely flow through the column (**Optional**: The Collection Tube may be emptied after Solution SR5 has completely flowed through the column. **Note**: DO NOT ALLOW THE COLUMN TO DRY OUT PRIOR TO LOADING THE RNA ISOLATION SAMPLE.)

14. Add the RNA Isolation Sample from Step 12 onto the **RNA Capture Column** and allow it to gravity flow through the column. Collect the flow through in the 15 ml Collection Tube.

15. Wash the column with 1 ml of **Solution SR5**. Allow it to gravity flow and collect the flow through in the 15 ml Collection Tube.

16. Transfer the **RNA Capture Column** to a new **15 ml Collection Tube** (provided). Shake Solution SR6 to mix and then add 1 ml of **Solution SR6** to the **RNA Capture Column** to elute the bound RNA into the **15 ml Collection Tube**. Allow **Solution SR6** to gravity flow into the **15 ml Collection Tube**.
    **Note**: The RNA PowerSoil® DNA Elution Accessory Kit is available for DNA elution (MO BIO Catalog# 12867-25). See the DNA Elution Procedure in the Hints and Troubleshooting Guide or contact MO BIO for details at technical@mobio.com.

17. Transfer the eluted RNA to a **2.2 ml Collection Tube** (provided) and add 1 ml of **Solution SR4**. Invert at least once to mix and incubate at -20°C for a minimum of 10 minutes.

18. Centrifuge the **2.2 ml Collection Tube** at 13,000 x *g* for 15 minutes at room temperature to pellet the RNA.

19. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 minutes to air dry the pellet.

20. Resuspend the RNA pellet in 100 µl of **Solution SR7**. For information on removal of genomic DNA from RNA, see the Hints and Troubleshooting Guide on page 13.

**Thank you for choosing the RNA PowerSoil® Total RNA Isolation Kit.**

***Appendix 12: QIAprep® Spin Miniprep kit protocol***

**Quick-Start**Protocol

# QIAprep® Spin Miniprep Kit

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAprep Miniprep Handbook, December 2006*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

**Notes before starting**

- **Optional**: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
- Add the provided RNase A solution to Buffer P1, mix, and store at 2–8°C.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 13,000 rpm (~17,900 x g) in a conventional table-top microcentrifuge.

1. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.

**October 2010**

QIAGEN®

Sample & Assay Technologies

6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pipetting. ▲ Centrifuge for 30–60 s and discard the flow-through, or ● apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB. ▲ Centrifuge for 30–60 s and discard the flow-through, or ● apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

   **Note**: This step is only required when using *endA*$^+$ strains or other bacteria strains with high nuclease activity or carbohydrate content.

8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. ▲ Centrifuge for 30–60 s and discard the flow-through, or ● apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source. Transfer the QIAprep spin column to the collection tube.

9. Centrifuge for 1 min to remove residual wash buffer.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.



***Appendix 13: pGEM-T system protocol***

Promega

**3. Protocol for Ligations Using the pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer**

**3.A. Ligation Protocol**

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as described below.

   **Note:** Use 0.5ml tubes known to have low DNA-binding capacity (e.g., VWR Cat.# 20170-310).

   Vortex the 2X Rapid Ligation Buffer vigorously before each use.
3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature.

   Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

| Reaction Component | Standard Reaction | Positive Control | Background Control |
|---|---|---|---|
| 2X Rapid Ligation Buffer, T4 DNA Ligase | 5µl | 5µl | 5µl |
| pGEM®-T or pGEM®-T Easy Vector (50ng) | 1µl | 1µl | 1µl |
| PCR product | Xµl* | – | – |
| Control Insert DNA | – | 2µl | – |
| T4 DNA Ligase (3 Weiss units/µl) | 1µl | 1µl | 1µl |
| nuclease-free water to a final volume of | **10µl** | **10µl** | **10µl** |

*Molar ratio of PCR product:vector may require optimization.

**Notes:**

1. Use only the T4 DNA Ligase supplied with this system to perform pGEM®-T and pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.
2. 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.
3. Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.

4. An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281). Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products, and to improve ligation efficiency. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers.

### 3.B. Optimizing Insert:Vector Molar Ratios

The pGEM®-T and pGEM®-T Easy Vector Systems have been optimized using a 1:1 molar ratio of the Control Insert DNA to the vectors. However, ratios of 8:1 to 1:8 have been used successfully. If initial experiments with your PCR product are suboptimal, ratio optimization may be necessary. Ratios from 3:1 to 1:3 provide good initial parameters. The concentration of PCR product should be estimated by comparison to DNA mass standards on a gel or by using a fluorescent assay (3). The pGEM®-T and pGEM®-T Easy Vectors are approximately 3kb and are supplied at 50ng/µl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

**Example of insert:vector ratio calculation:**

How much 0.5kb PCR product should be added to a ligation in which 50ng of 3.0kb vector will be used if a 3:1 insert:vector molar ratio is desired?

$$\frac{50\text{ng vector} \times 0.5\text{kb insert}}{3.0\text{kb vector}} \times \frac{3}{1} = 25\text{ng insert}$$

Using the same parameters for a 1:1 insert:vector molar ratio, 8.3ng of a 0.5kb insert would be required.

**Tip:** The Biomath calculator (**www.promega.com/biomath/**) can be used to determine the amount of insert DNA needed. The pGEM®-T Vector size is 3000bp and the pGEM®-T Easy Vector size is 3015bp.

***Appendix 14: QIAquick PCR Purification kit protocol***

**Quick-Start**Protocol

# QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAquick Spin Handbook, March 2008*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

**Notes before starting**

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ▲ a provided 2 ml collection tube or into ● a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ▲ centrifuge for 30–60 s or ● apply vacuum to the manifold until all the samples have passed through the column. ▲ Discard flow-through and place the QIAquick column back in the same tube.

**October 2010**

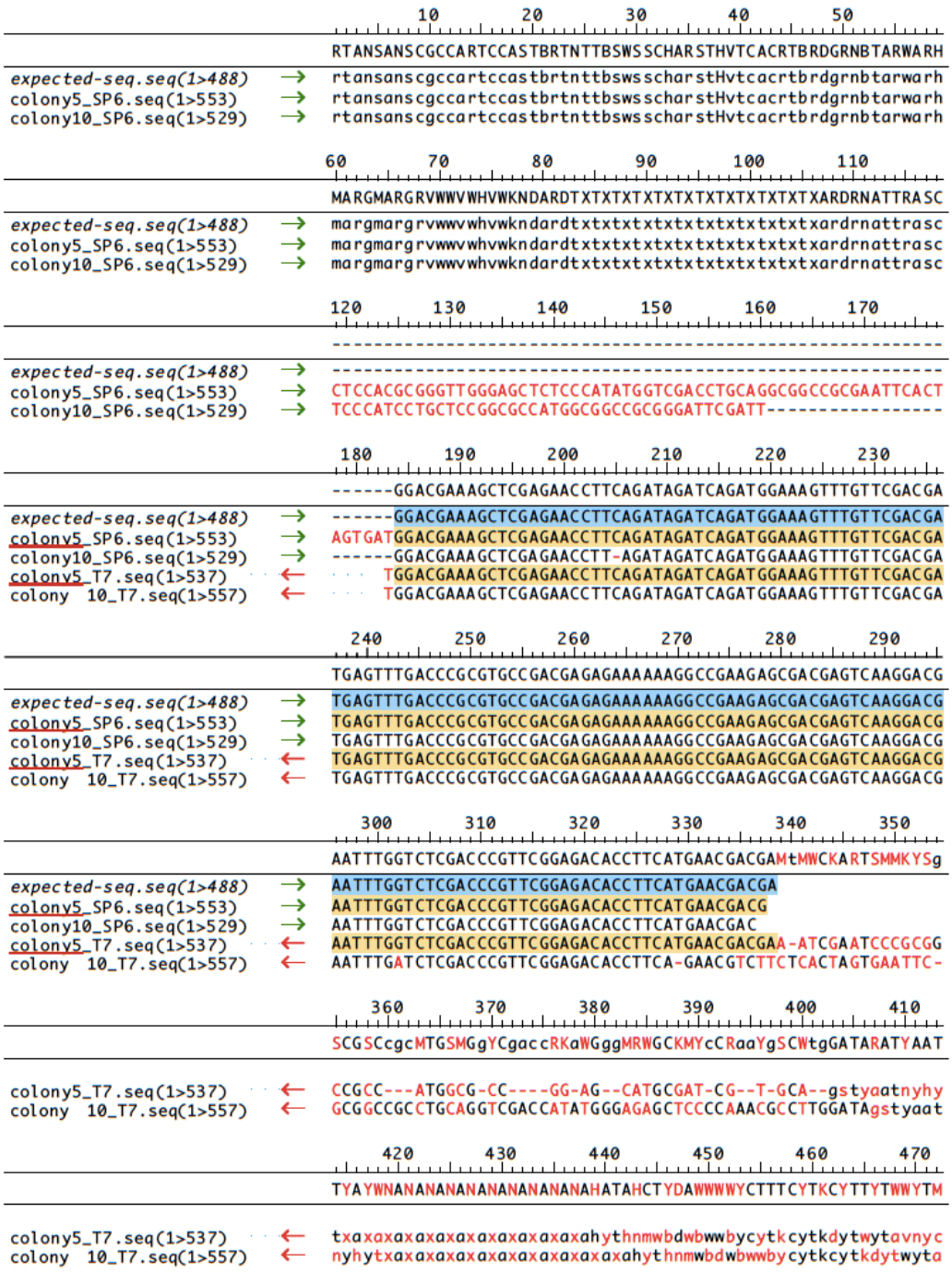
QIAGEN®

Sample & Assay Technologies

4. To wash, add 0.75 ml Buffer PE to the QIAquick column ▲ centrifuge for 30–60 s or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.



***Appendix 15: Sequences alignement of the inserts CDP6-x7 in pGEM-T Easy***

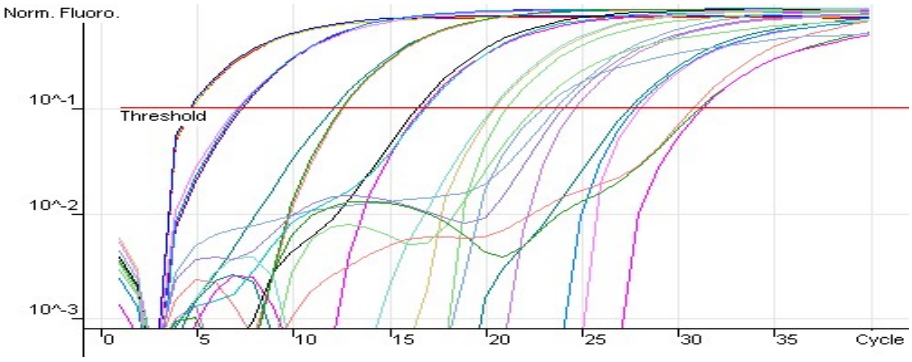


***Appendix 16: qPCR results for the characterization of pASI standard curve***

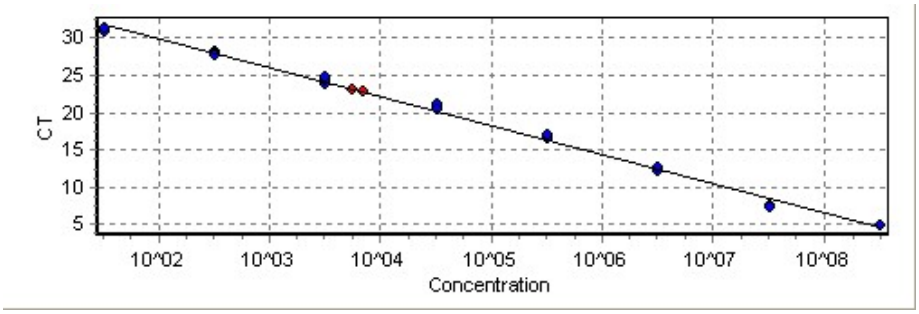
**Quantitaton Information**

| | |
|---|---|
| Threshold | 0.10404 |
| Left Threshold | 1.000 |
| Standard Curve Imported | No |
| Standard Curve(1) | conc=10^(-0.257*CT+9.691) |
| Standard Curve(2) | CT=-3.889*log(conc)+37.687 |
| Reaction efficiency(*) | 0.8078(*=10^(-1/m)-1) |
| M | -3.88879 |
| B | 37.68738 |
| R Value | 0.99776 |
| R^2 Value | 0.99553 |
| Start normalizing from cycle | 1 |
| Noise Slope Correction | No |
| Reaction Efficiency Threshold | Disabled |
| Normalisation Method | Standard |
| Digital Filter | Light |
| No Template Control Threshold | 27% |
| Sample Page | Page1 |

**Quantitation data for CyclingA.FAM/Sybr**




**Standard Curve**



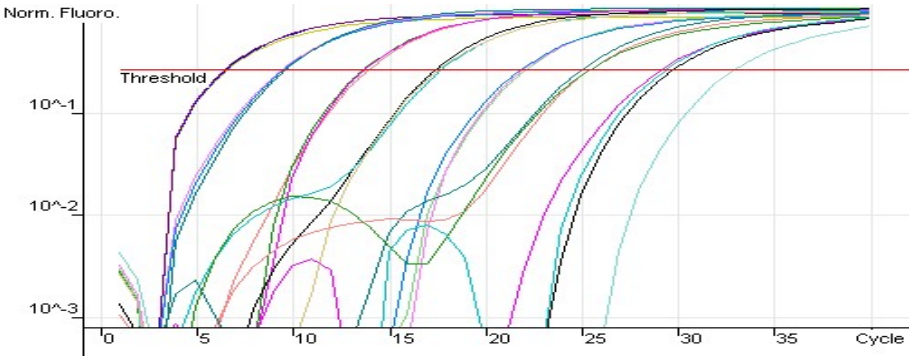
| No. | Name | Type | Ct | GivenConc(copies/ul) | CalcConc(copies/ul) | %Var |
|---|---|---|---|---|---|---|
| A1 | pASI-1 | Standard | 4.87 | 326026426 | 275423096 | 15.5% |
| A2 | pASI-1 | Standard | 4.83 | 326026426 | 281928157 | 13.5% |
| A3 | pASI-1 | Standard | 4.74 | 326026426 | 297518148 | 8.7% |
| A4 | pASI-2 | Standard | 7.46 | 32602643 | 59160748 | 81.5% |
| A5 | pASI-2 | Standard | 7.26 | 32602643 | 66813855 | 104.9% |
| A6 | pASI-2 | Standard | 7.33 | 32602643 | 63946173 | 96.1% |
| A7 | pASI-3 | Standard | 12.18 | 3260264 | 3633782 | 11.5% |
| A8 | pASI-3 | Standard | 12.69 | 3260264 | 2679813 | 17.8% |
| B1 | pASI-3 | Standard | 12.60 | 3260264 | 2820786 | 13.5% |
| B2 | pASI-4 | Standard | 16.84 | 326026 | 230082 | 29.4% |
| B3 | pASI-4 | Standard | 16.56 | 326026 | 270949 | 16.9% |
| B4 | pASI-4 | Standard | 16.98 | 326026 | 211518 | 35.1% |
| B5 | pASI-5 | Standard | 20.61 | 32603 | 24612 | 24.5% |
| B6 | pASI-5 | Standard | 21.21 | 32603 | 17232 | 47.1% |
| B7 | pASI-5 | Standard | 20.47 | 32603 | 26709 | 18.1% |
| B8 | pASI-6 | Standard | 23.79 | 3260 | 3744 | 14.9% |
| C1 | pASI-6 | Standard | 24.19 | 3260 | 2959 | 9.2% |
| C2 | pASI-6 | Standard | 24.76 | 3260 | 2113 | 35.2% |
| E5 | pASI-7 | Standard | 28.14 | 326 | 285 | 12.5% |
| E6 | pASI-7 | Standard | 27.88 | 326 | 333 | 2.2% |
| E7 | pASI-7 | Standard | 27.60 | 326 | 394 | 20.7% |
| E8 | pASI-8 | Standard | 30.92 | 33 | 55 | 66.3% |
| F1 | pASI-8 | Standard | 31.26 | 33 | 45 | 36.3% |
| F2 | pASI-8 | Standard | 31.39 | 33 | 42 | 26.2% |
| F3 | pASI-9 | Standard | | 3 | | |
| F4 | pASI-9 | Standard | | 3 | | |
| F5 | pASI-9 | Standard | | 3 | | |

***Appendix 17: qPCR results for the characterization of pASP standard curve***

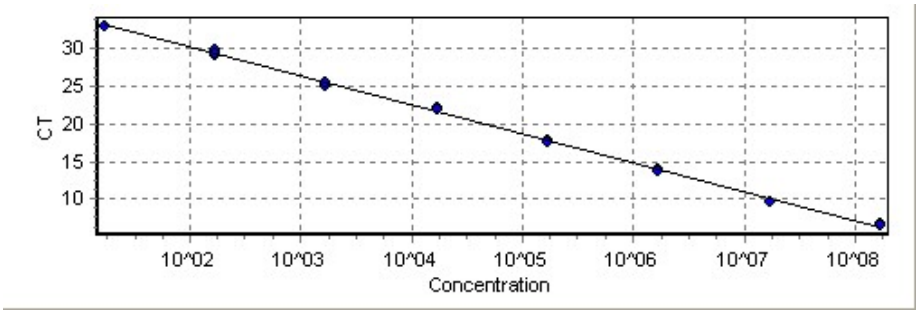
**Quantitaton Information**

| | |
|---|---|
| Threshold | 0.2689 |
| LeftThreshold | 1.000 |
| StandardCurveImported | No |
| StandardCurve(1) | conc=10^(-0.259*CT+9.835) |
| StandardCurve(2) | CT=-3.865*log(conc)+38.012 |
| Reactionefficiency(*) | 0.81435(*=10^(-1/m)-1) |
| M | -3.86516 |
| B | 38.01217 |
| RValue | 0.99922 |
| R^2Value | 0.99843 |
| Startnormalisingfromcycle | 1 |
| NoiseSlopeCorrection | No |
| ReactionEfficiencyThreshold | Disabled |
| NormalisationMethod | Standard |
| DigitalFilter | Light |
| NoTemplateControlThreshold | 27% |
| SamplePage | Page1 |

**Quantitation data for CyclingA.FAM/Sybr**




**Standard Curve**



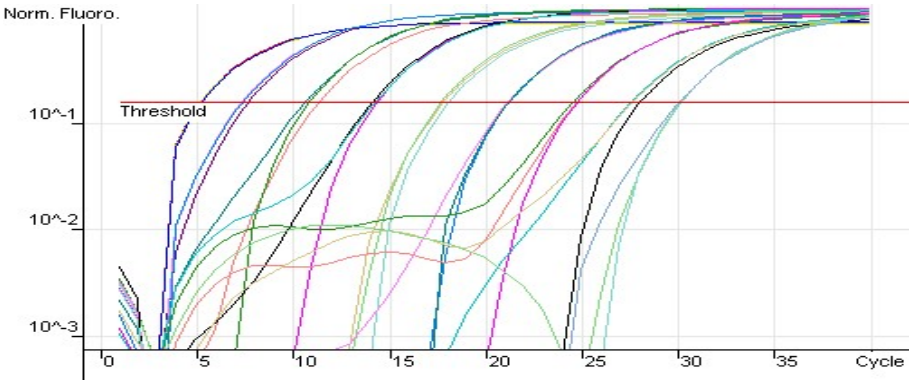
| No. | Name | Type | Ct | GivenConc(Copies) | CalcConc(Copies) | %Var |
|---|---|---|---|---|---|---|
| A1 | NTC | NTC | | | | |
| A2 | pASP-1 | Standard | 6.71 | 168827939 | 125677131 | 25.6% |
| A3 | pASP-1 | Standard | 6.60 | 168827939 | 133732915 | 20.8% |
| A4 | pASP-1 | Standard | 6.55 | 168827939 | 137982995 | 18.3% |
| A5 | pASP-2 | Standard | 9.50 | 16882794 | 23824007 | 41.1% |
| A6 | pASP-2 | Standard | 9.62 | 16882794 | 22193314 | 31.5% |
| A7 | pASP-2 | Standard | 9.70 | 16882794 | 21092518 | 24.9% |
| A8 | pASP-3 | Standard | 14.02 | 1688279 | 1615328 | 4.3% |
| B1 | pASP-3 | Standard | 13.74 | 1688279 | 1908959 | 13.1% |
| B2 | pASP-3 | Standard | 13.79 | 1688279 | 1844430 | 9.2% |
| B3 | pASP-4 | Standard | 17.52 | 168828 | 200809 | 18.9% |
| B4 | pASP-4 | Standard | 17.83 | 168828 | 166397 | 1.4% |
| B5 | pASP-4 | Standard | 17.66 | 168828 | 184088 | 9.0% |
| B6 | pASP-5 | Standard | 22.19 | 16883 | 12399 | 26.6% |
| E5 | pASP-5 | Standard | 22.07 | 16883 | 13332 | 21.0% |
| E6 | pASP-5 | Standard | 21.92 | 16883 | 14567 | 13.7% |
| E7 | pASP-6 | Standard | 25.10 | 1688 | 2191 | 29.8% |
| E8 | pASP-6 | Standard | 25.52 | 1688 | 1705 | 1.0% |
| F1 | pASP-6 | Standard | 25.54 | 1688 | 1690 | 0.1% |
| F2 | pASP-7 | Standard | 29.23 | 169 | 187 | 10.4% |
| F3 | pASP-7 | Standard | 29.85 | 169 | 129 | 23.4% |
| F4 | pASP-7 | Standard | 29.49 | 169 | 161 | 4.9% |
| F5 | pASP-8 | Standard | | 17 | | |
| F6 | pASP-8 | Standard | | 17 | | |
| F7 | pASP-8 | Standard | 33.06 | 17 | 19 | 12.2% |
| F8 | pASP-9 | Standard | | 2 | | |
| G1 | pASP-9 | Standard | | 2 | | |
| G2 | pASP-9 | Standard | | 2 | | |

***Appendix 18: qPCR results for the characterization of pASC standard curve***

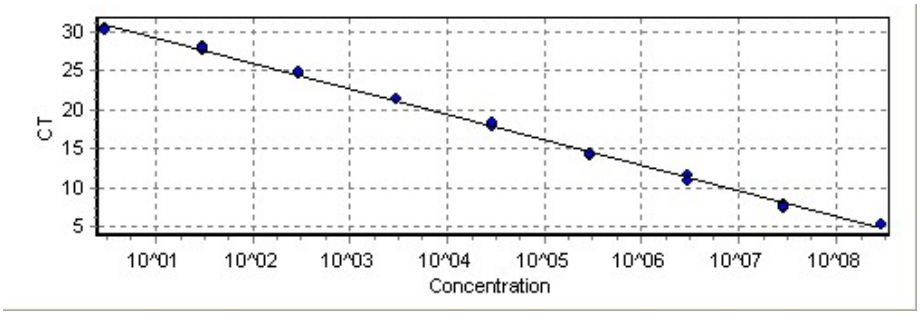
**Quantitaton Information**

| | |
|---|---|
| Threshold | 0.1589 |
| Left Threshold | 1.000 |
| Standard Curve Imported | No |
| Standard Curve (1) | conc= 10^(-0.308*CT + 9.957) |
| Standard Curve (2) | CT = -3.250*log(conc) + 32.363 |
| Reaction efficiency (*) | 1.03078 (* = 10^(-1/m) - 1) |
| M | -3.25031 |
| B | 32.36291 |
| R Value | 0.9988 |
| R^2 Value | 0.9976 |
| Start normalising from cycle | 1 |
| Noise Slope Correction | No |
| Reaction Efficiency Threshold | Disabled |
| Normalisation Method | Standard |
| Digital Filter | Light |
| No Template Control Threshold | 27% |
| Sample Page | Page 1 |

**Quantitation data for Cycling A.FAM/Sybr**




**Standard Curve**



| No. | Name | Type | Ct | Given Conc (copies/ul) | Calc Conc (copies/ul) | % Var |
|---|---|---|---|---|---|---|
| A1 | pASC-1 | Standard | 5.23 | 297 916 519.00 | 222 352 397.72 | 25.4% |
| A2 | pASC-1 | Standard | 5.27 | 297 916 519.00 | 216 273 732.55 | 27.4% |
| A3 | pASC-1 | Standard | 5.30 | 297 916 519.00 | 211 606 482.13 | 29.0% |
| A4 | pASC-2 | Standard | 7.80 | 29 791 652.00 | 36 188 700.58 | 21.5% |
| A5 | pASC-2 | Standard | 7.44 | 29 791 652.00 | 46 599 372.31 | 56.4% |
| A6 | pASC-2 | Standard | 7.51 | 29 791 652.00 | 44 305 257.90 | 48.7% |
| A7 | pASC-3 | Standard | 10.75 | 2 979 165.00 | 4 455 065.35 | 49.5% |
| A8 | pASC-3 | Standard | 11.45 | 2 979 165.00 | 2 717 299.63 | 8.8% |
| B1 | pASC-3 | Standard | 10.94 | 2 979 165.00 | 3 902 487.63 | 31.0% |
| B2 | pASC-4 | Standard | 14.44 | 297 917.00 | 326 161.98 | 9.5% |
| B3 | pASC-4 | Standard | 14.20 | 297 917.00 | 387 315.27 | 30.0% |
| B4 | pASC-4 | Standard | 14.36 | 297 917.00 | 344 790.42 | 15.7% |
| B5 | pASC-5 | Standard | 17.95 | 29 792.00 | 27 195.39 | 8.7% |
| B6 | pASC-5 | Standard | 17.82 | 29 792.00 | 29 850.92 | 0.2% |
| B7 | pASC-5 | Standard | 18.20 | 29 792.00 | 22 741.55 | 23.7% |
| E5 | pASC-6 | Standard | 21.30 | 2 979.17 | 2 538.78 | 14.8% |
| E6 | pASC-6 | Standard | 21.35 | 2 979.17 | 2 447.35 | 17.9% |
| E7 | pASC-6 | Standard | 21.33 | 2 979.17 | 2 474.71 | 16.9% |
| E8 | pASC-7 | Standard | 24.86 | 297.92 | 203.86 | 31.6% |
| F1 | pASC-7 | Standard | 24.65 | 297.92 | 236.78 | 20.5% |
| F2 | pASC-7 | Standard | 24.85 | 297.92 | 204.47 | 31.4% |
| F3 | pASC-8 | Standard | 28.13 | 29.79 | 20.10 | 32.5% |
| F4 | pASC-8 | Standard | 27.67 | 29.79 | 27.87 | 6.4% |
| F5 | pASC-8 | Standard | 27.73 | 29.79 | 26.54 | 10.9% |
| F6 | pASC-9 | Standard | 30.25 | 2.98 | 4.47 | 49.9% |
| F7 | pASC-9 | Standard | 30.22 | 2.98 | 4.57 | 53.5% |
| F8 | pASC-9 | Standard | 30.16 | 2.98 | 4.75 | 59.4% |
| G1 | NTC | NTC | | | | |
| G2 | Oligo + | Unknown | | | | |
| G3 | Oligo - | Unknown | | | | |

This report generated by Rotor-Gene Real-Time Analysis Software 6.0 (Build 14)